Selection of novel antigens from *Leishmania* spp. and design of live recombinant salmonella vaccines against experimental visceral leishmaniasis

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Abbreviations

AIDA	Autotransporter involved in diffuse adherence
AIDS	Acquired immunodeficiency syndrome
AmpR	Ampicillin resistance
APC	Allophycocyanin
APC	Antigen presenting cell
APS	Ammonium persulfate
BCG	Bacillus of Calmette and Guerin
bla	beta-lactamase
BMI	Body-mass index
BSA	Bovine serum albumin
CAI	Codon adaptation index
CD	Cluster of differentiation
CFU	colony forming unit
CL	cutaneous leishmaniasis
CLA	Cutaneous lymphocyte antigen
cm	centimeter
CMV	Cytomegalovirus
CTB-SP	cholera toxin B signal peptide
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CV	column volumes
DAPI	Diamidino-2-phenylindole
DC	Dendritic cell
ddH_2O	didestilled water
°C	degree centrigrade
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates
DTH	Delayed-type hypersensitivity
E.	*Escherichia*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
ELISA	Enzyme linked immunosorbant assay
et al.	et alii
etc	et cetera
EtOH	Ethanol
FACS	Fluorescent cell sorting
FCS	Fetal calf serum
fig	figure
FPLC	Fast performance liquid chromatography
FT	flow through
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp63	Glycoprotein 63
h	Hour
H.	*Helicobacter*
HA	*Haemophilus influenzae* hemaagglutinin
HASPB1	Hydrophilic acylated surface protein B1
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

HRP	Horseradish peroxidase
Hsp60	Heat shock protein
ICS	Intracellular cytokine staining
i.e.	id est (that is)
IFN	Interferon
i.g.	intra gastric
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intra peritoneal
IPTG	Isopropyl-β-D-thiogalactopyranoside
i.v.	intra venous
KDa	Kilodalton
KMP-11	Kinetoplastid membrane protein
L.	*Leishmania*
L (l)	litre
LACK	Leishmania homologue of receptors for activated C kinase
LB	Luria-Bertani
LDU	Leishman-Donovan unit
LeIF	Leishmania elongation initiation factor
LmSTI1	*Leishmania major* stress-inducible protein 1
LPG	Lipophospoglycan
LPS	Lipopolysaccharide
Lys	lysate
m	meter
m	milli
M	molar
Mφ	Macrophage
MCS	multiple cloning site
MEM	Minimum essential medium
MHC	major histocompatibility complex
MIF	Migration inhibitory factor
min	Minutes
MOPS	3-(N-morpholino) propanesulfonic acid
MPL-SE	Monophosphoryl lipid A-stable emulsion
mRNA	messenger ribonucleic acid
MRP2	mitochondrial RNA binding protein 2
µ	micro
n	nano
N.	*Neisseria*
NK cells	Natural killer cells
NRAMP1	Natural-resistance-associated macrophage protein
OD	Optical density
OMV	Outer membrane vesicle
orf	Open reading frame
ori	Origin of replication
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PerCP	Peridinin chlorophyll protein complex
PKDL	post kala-azar dermal leishmaniasis
PMA	Phorbol 12-myristate 13-acetate

PSA-2	Promastigote surface antigen 2
PVDF	Polyvinylidene fluoride
RBS	Ribosomal binding site
rpm	rounds per minute
s	seconds
s.c.	sub cutaneous
S.	*Salmonella*
SDM	semi defined media
SDS	Sodium dodecyl sulfate
SPI-2	*Salmonella* pathogenicity island 2
TBE	Tris borate EDTA
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLR	Toll like receptor
TNF	Tumor necrosis factor
TSA	Thiol-specific-antioxidant
T3SS	Type III secretions system
TRYP	Tryparedoxin peroxidase
UK	United Kingdom
US$	US dollar
UV	ultraviolet
V.	*Vibrio*
v/v	volume per volume
V	Volt
VL	visceral leishmaniasis
w/v	weight per volume
WHO	World Health Organisation
xg	x times acceleration of gravity
%	percent

Zusammenfassung

Leishmaniosen gehören zu den tropischen Krankheiten und bedrohen geschätzte 350 Millionen Menschen in 88 Ländern weltweit. Die schwerste Form, viszerale Leishmaniose, betrifft die ärmsten Bevölkerungsschichten und ist die Ursache für circa 50 000 Todesfälle pro Jahr. Es wird angenommen, dass die Entwicklung eines Impfstoffs möglich ist, aber trotz aller Bemühungen, steht derzeit noch kein Impfstoff zur Verfügung.

Im Rahmen dieser Arbeit wurde ein Impfstoff gegen viszerale Leishmaniose entwickelt und *in vivo* auf pre-klinischer Ebene getestet. Des Weiteren wurden rekombinante Membranvesikel konstruiert, um ein Boostreagenz zu erhalten. Die Herstellung sowohl des rekombinanten Salmonellenimpfstoffs als auch der Membranvesikel sollte, trotz des geringen Handelspreis, ökonomisch praktikabel sein, was besonders wichtig ist für Menschen in den betroffenen Entwicklungsländern.

Der erste Schritt war die Auswahl neuartiger Antigenkandidaten aus einem Proteomics Datensatz, in dem beide *Leishmania* Lebensformen verglichen wurden. Der Schwerpunkt wurde auf abundante, hypothetische Proteine gelegt, die sowohl in Pro- als auch Amastigoten identifiziert wurden, in Leishmanienarten hochkonserviert sind aber gleichzeitig keine Sequenzhomologien zu humanen und murinen Proteinen besitzen. Diese Antigene wurden in unterschiedlicher Menge auf der Oberfläche und im Cytoplasma von *S. typhimurium* SL3261 und auch auf Membranvesikeln exprimiert. Impfstämme wurden selektiert in Hinsicht auf ihre bakterielle Fitness und Antigenexpression.

Es konnte gezeigt werden, dass LinJ08.1140-, LinJ23.0410-exprimierende Impfstämme oder eine Mischung dieser in der Lage waren besonders anfällige BALB/c Mäuse vor *L. major* und wichtiger *L. donovani* Infektion zu schützen. Analyse der humoralen Immunantwort deutet darauf hin, dass der Impfschutz das Ergbnis einer T_H1 Antwort war. Erste Schritte zur Aufklärung struktureller und funktioneller Eigenschaften von LinJ08.1140 wurden unternommen. Es wird allgemein angenommen, dass antigenspezifische $CD4^+$ und $CD8^+$ T-Zellen am Schutz beteiligt sind. Daher wurde für LinJ08.1140 potentielle MHC-I Epitope mit Hilfe von bioinformatischen Programmen vorhergesagt. Zusätzlich deuten Fluoreszenz-färbungen mit antigenspezifischen Antikörpern in *L. major* Promastigoten darauf hin, dass LinJ08.1140 eine Rolle bei der Zellteilung spielt.

Summary

Leishmaniasis is a neglected tropical disease and currently an estimated 350 million people in 88 countries around the world are at risk. Its most severe form, visceral leishmaniasis, affects the poorest people in a population and causes an estimated 50 000 deaths every year. Vaccination is thought to be feasible but despite all efforts, no vaccine is yet available. Vaccines will mainly be targeted for people in developing countries such as India, thus focus has to be placed on affordability.

In this thesis a vaccine against visceral leishmaniasis was designed and evaluated *in vivo* at pre-clinical level. Furthermore, recombinant outer membrane vesicles were developed in an attempt to create a booster reagent. Both, the recombinant salmonella vaccine and the preparation of outer membrane vesicles should be commercially viable, and can still be sold at low prices, which is crucial for people in developing countries.

First, novel antigen candidates were selected using proteomics data comparing leishmania life stages. Abundant and hypothetic proteins, which have been identified in both parasite life stages and have high sequence homology throughout *Leishmania* species while lacking homologues in human and mouse, were selected. These antigens were differentially expressed on the surface or in the cytosol of *S. typhimurium* SL3261 and in the form of outer membrane vesicles. A two step procedure was developed to select optimised vaccine strains based on bacterial fitness and antigen expression.

Selected salmonella strains expressing LinJ08.1140, LinJ23.0410 or an admixture of these strains are shown to protect susceptible BALB/c mice by reducing visceralisation of *L. major* and more importantly *L. donovani* infections. Analysis of vaccine specific antibody responses suggests that protection resulted from induction of a T_H1 response. First steps were undertaken towards resolving functional and structural properties of the most protective antigen LinJ08.1140. Putative MHC-I epitopes of antigen LinJ08.1140 were predicted using bioinformatics since antigen-specific $CD4^+$ and $CD8^+$ T cells are believed to be required. In addition, immunofluorescent staining of LinJ08.1140 in *L. major* promastigotes suggested a functional role for this antigen in parasite cell division, since especially dividing cells emmited a strong fluorescence signal.

1. Introduction

1.1 Leishmania life cycle

Leishmania are uniflagelated protozoan parasites which belong to the class of *Kinetoplastida*, order of *Trypanosomatid*a and the genus *Leishmania*. These parasites have a digenic life cycle, involving two hosts, the sand fly vector and the vertebrate host. The parasites are transmitted by the bite of infected female sand flies of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World).

Two life forms of Leishmania parasites are existent, which differ highly in appearance. Immotile amastigotes are mainly found in mammalian host cells and are of 2-4 µm oval shape. Promastigotes are larger, measuring up to 20 µm and have a flagellum which makes them highly motile. They reside predominantly in the sand fly vector.

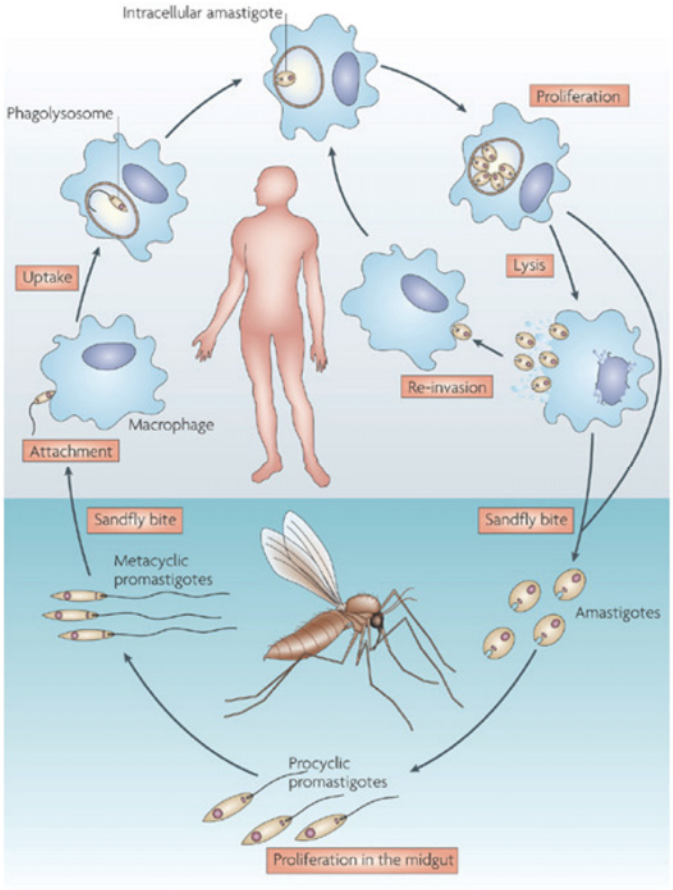

Fig. 1.1: Digenic life cycle of leishmania species

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Female sand flies need blood as a protein source for egg development. After uptake of blood from infected mammals, leishmania parasites develop in the midgut of the sand fly where they undergo major transformation from amastigote to promastigote states. After proliferation and maturation, highly infective metacyclic promastigotes move to the salivary glands of the sand fly, from where they are transmitted during the blood meal into mammalian hosts. In the skin parasites are ingested by neutrophils, which then undergo apoptosis and are subsequently taken up by macrophages for disposal. This has been shown for *L. major* (reviewed by Peters and Sacks, 2009; Laskay *et al*., 2008; Ritter *et al*., 2009) as well as *L. donovani* (Gueirard *et al*., 2008). In macrophages parasites evade killing, transform into amastigotes and begin to proliferate. Highly infected macrophages burst and release amastigotes which in turn infect more macrophages. During the blood meal from another sand fly amastigotes residing in macrophages are taken up and development into promastigotes commences again.

1.2 Leishmania related diseases

Early descriptions of leishmania disease date back to 15th and 16th century, when seasonal agricultural workers returned from the Andes with ulcers. Due to these disfigurements the disease was called “white leprosy”. In 1901 William Leishman identified organism in smears from a spleen of a patient who died from Dum-Dum fever. Two years later, in 1903, Captain Charles Donovan described these organisms as a new species. The link between parasites and disease was finally established by Major Ronald Ross, who named the new species after its two discoverers *Leishmania donovani* (www.who.int/leishmaniasis/history_disease/en/index.html, 2009).

To date more than 20 Leishmania species have been identified as the causative agent to a wide spectrum of human diseases. Currently an estimated 12 million people are infected, while 350 million people in 88 countries from all over the world are at risk to develop one of the diseases associated with *Leishmania* parasites (www.who.int/leishmaniasis/disease_epidemiology/en/index.html, 2009; (Desjeux, 1996).

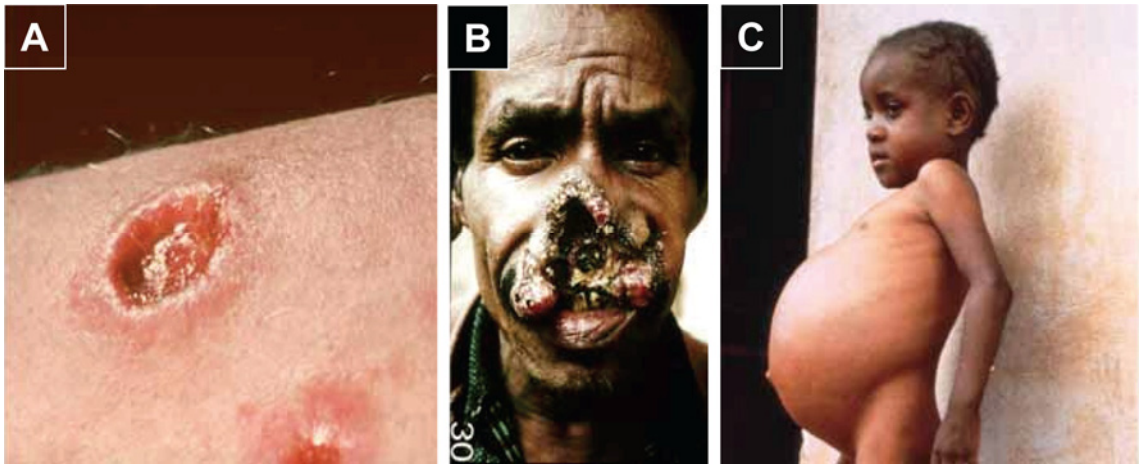

Fig. 1.2: Leishmania species cause a wide spectrum of diseases

A: skin lesion from cutaneous leishmaniasis, **B**: mucocutaneous leishmaniasis, **C**: visceral leishmaniasis

1.2.1 Cutaneous leishmaniasis

Leishmaniasis of the skin is caused by parasites belonging to the *L. (vianna) brasiliensis* and *L. mexicana* complex in the New World and *L. major* and *L. tropica* in the Old World. It is mainly a zoonotic disease, the reservoir ranging from small rodents (e.g. gerbils) to bigger mammals (e.g. opossum, sloth), but can be anthroponotic in case of *L. tropica*. An occurrence of 1-1.5 million new cases every year has been estimated. The disease affects mainly the skin of exposed areas (e.g. face, arms and legs) and can remain sub-clinical. After several weeks first symptoms occur with the development of papules which progress to nodules and later to lesions (fig. 1.2A). These lesions are normally painless and heal spontaneously after several months, often resulting in scars and disfigurements. Further complications include the development of satellite lesions, disseminating to the lymph nodes and even visceralisation.

Mucocutanous leishmaniasis, also known as espundia is a dreaded complication of cutaneous leishmaniasis (CL) and leads to the progressive destruction of mucosal tissue by the parasite. It is mainly caused by *L. braziliensis* and *L. panamensis*.

1.2.2 Visceral leishmaniasis

The most severe form is visceral leishmaniasis (VL), also known as Dum-Dum fever or kala-azar in India and can be found in Latin America, the Mediterranean Basin and Asia. An estimated 500 000 new cases and 50 000 deaths are reported every year and 90 % from countries like Bangladesh, Nepal, India, Sudan, Ethiopia and Brazil (Chappuis *et al*., 2007). This systemic disease is caused by *L. chagasi* in

Fig. 1.3: Distribution of visceral leishmaniasis world wide

Regions highlighted in red show endemic areas of VL
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the New World and *L. infantum* and *L. donovani* in the Old World. VL is mainly zoonotic with dogs being the main reservoir, however, transmission in areas endemic for *L. donovani* (e.g. India and Sudan) is anthroponotic. In many cases infection remains asymptomatic, but dependant on circumstances can become acute or chronic. Then a sub-clinical incubation period between two and six month is followed by first symptoms like fever, fatigue, weight loss, weakness, which are accompanied by hepato-splenomegally (enlarged liver and spleen) and anaemia caused by internal bleeding. Untreated patients may die from bacterial co-infections, massive internal bleedings and anaemia (reviewed by Chappuis *et al*., 2007; Herwaldt, 1999).

Post kala-azar dermal leishmaniasis (PKDL) is a complication of VL which develops 0-6 month (Sudan) or 2-many years (India) after recovery. The disease presents itself

in different forms, mainly as a maculopapular, nodular or papular rash which spreads from face to the whole body, dependant on the grade of disease (Zijlstra *et al*., 2003). These rashes harbour high numbers of profoundly infective parasites which are serving as a reservoir for the anthroponotic cycle.

1.3 Treatment of visceral leishmaniasis

While CL normally heals spontaneously, VL can be fatal without treatment. Visceral leishmaniasis is a disease which affects the poorest populations. Olliaro and colleagues estimated the financial cost to manage one episode of visceral leishmaniasis for a household in India. Cost taken into account included lack of income due to illness, cost for medical treatment and transportation, which altogether averaged at 9420 rupies (209 US$). This accounts for more than four times the monthly average household income, which was estimated to be 2200 rupies (49 US$) (Olliaro *et al*., 2009).

Chemotherapeutic treatment is available, but due to high toxicity and adverse effects, lower doses are often administered, encouraging the emergence of parasite resistance (Polonio and Efferth, 2008). Pentavalent antimonials have been the treatment of choice for more than six decades. These are now considered as lost, due to the increased resistance of *L. donovani* especially in the Indian state of Bihar (Sundar *et al*., 2000; Lira *et al*., 1999). The replacement Amphothericin B deoxycholate is highly effective but also has intolerable toxic side effects including cardio- and nephrotoxicity and even death. The toxic deoxycholate part has been replaced with different liquid formulations, resulting in AmBisome®, which is a registered drug against VL in India. Although the price of the drug has been reduced from 200 US$ to 20 US$ per vial, treatment cost remain expensive, as a single dose is less effective. In addition a high relapse rate of 80-100 % has been found in immunosuppressed patients (Meyerhoff, 1999). Miltefosine is the first drug to be taken orally, reducing toxicity to mild or moderate symptoms. Major drawbacks are the prolonged treatment period, often enhancing failure due to inconsistencies in adherence to treatment plan, high drug cost (between 53 and 85 US$ per pack) and teratogenic effects of Miltefosine, contraindicating its use in pregnant women or generally women of child-bearing age. Its prolonged half-life is the reason for concerns regarding the emergence

of resistance (Olliaro and Sundar, 2009; Sundar and Chatterjee, 2006; Olliaro *et al*., 2009). A newly registered drug is Paromomycin, which is given as intra-muscular injections over a period of 21 days. Low drug cost of 10 US$ per adult and 5 US$ per child making this drug the cheapest available so far. In respect to the low household income, costs of treatment are still too high and will remain so in the foreseeable future.

1.4 Immunology of leishmaniases

The work of Mosmann and Coffman led to the T_H1/T_H2 paradigm for protection or susceptibility to intracellular pathogens (Mosmann *et al*., 1986; Stevens *et al*., 1988).
Figure 1.4 shows a simplified model of susceptibility and resistance in mice. However, while control of leishmaniases remains T_H1 (IL-12, IFNγ, TNFα, IL-2) dependant, there is compelling evidence for *L. major* and *L. donovani*, that typical T_H2 cytokines IL-4 and IL-13 can enhance protection by inducing IL-12 production in macrophages and dendritic cells (Basu *et al*., 2005; Murphy *et al*., 1998; Stager *et al*., 2003a; Hochrein *et al*., 2000; reviewed by Alexander and McFarlane, 2008; Alexander and Bryson, 2005). Furthermore, IFNγ and IL-4 producing T cells have been found in asymptomatic and cured patients. It had been speculated that enhanced protection is rather down to an increased frequency of cytokine producing T cells than an altered IFNγ/IL-4 balance (Murphy *et al*., 1998).
Another T_H2 cytokine, IL-10 has been shown to promote parasite residence and multiplication in resistant and susceptible mice. Interleukin-10 has anti-inflammatory properties and is produced by a wide range of cells, e.g. macrophages, dendritic cells, B cells, T cells and epithelial cells. It has been found in high levels in patients with VL, where it is thought to limit immune mediated pathogenesis by inhibition and down regulation of IL-12 and MHC-II, thus promoting parasite replication and disease progression (reviewed by Nylen and Sacks, 2007).
Furthermore IL-10 has been shown to promote B cell survival, resulting in hypergammaglobulinaemia and isotype class switch to IgG1 and IgG3 (Caldas *et al*., 2005). A contribution of leishmania-specific IgG in disease promotion has been shown (Miles *et al*., 2005) and hypergammaglobulinaemia is a common parameter in patients with active VL. It has been found that the sources of disease promoting IL-10

are rather antigen driven Tr1 cells than natural Tregs (reviewed by Nylen and Sacks, 2007).

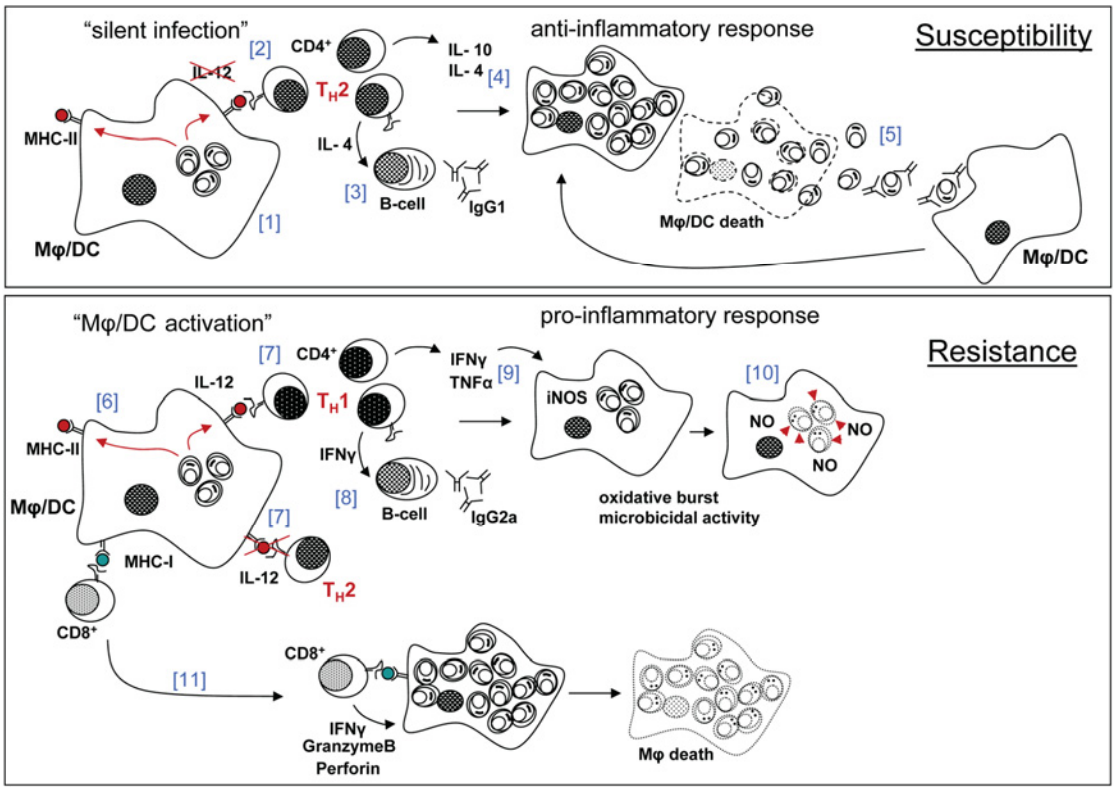

Fig. 1.4: Simplified overview of the leishmania immuno-biology paradigm based on the murine L. major infection model

Upper panel shows scenario in susceptible mice. [**1**] non-activated infected APC presents leishmania antigens via MHC-II to $CD4^+$ T cells, [**2**] the lack of IL-12 leads to activation of T_H2 T cells and production of anti-inflammatory cytokines [**3**] IL-4 secretion induces antibody class switch to IgG1 [**4**] anti-inflammatory cytokines promote uncontrolled parasite multiplication leading to the burst of the host cell [**5**] release of amastigotes, followed by opsonization with IgG1 and Fcγ mediated uptake by uninfected APCs.

Lower panel shows scenario in resistant mice. [**6**] upon infection APC become activated and present parasite antigens via MHC-II to $CD4^+$ T cells, [**7**] presence of IL-12 leads to activation of T_H1 T cells and inhibition of T_H2, [**8**] IFNγ secretion promotes antibody class switch to IgG2a, [**9**] pro-inflammatory cytokines up-regulate among others iNOS, [**10**] mechanism including oxidative burst and microbicidal activities lead to the killing of the parasite by the host, [**11**] MHC-I presentation leads to activation of cytotoxic T cells ($CD8^+$) which can kill the infected host cell along with the parasites.

Abbreviations: MHC major histocompatibility complex, IL interleukin, Ig immunoglobulin, CD cluster of differentiation, iNOS inducible nitric oxide synthase, IFN interferon, DC dendritic cell, Mφ macrophage, APC antigen presenting cell

Recent vaccine studies also suggested the contribution of cytotoxic $CD8^+$ T cells in vaccine induced protection against experimental visceral leishmaniasis in mice (Basu *et al*., 2007a; Murray *et al*., 1989; Stager *et al*., 2003b).
Genetic factors also play a role in disease progression. Natural-resistance-associated macrophage protein (NRAMP1) has been shown to limit the replication of intracellular pathogens and susceptibility against visceral leishmaniasis is linked to polymorphism in the NRAMP1 gene (Mohamed *et al*., 2004).

1.5 Leishmaniases – a growing problem?

Several factors have been singled out to contribute to the spreading of leishmaniases world wide, causing present and future problems and underlining the need for a vaccine.
The main reservoirs for the parasites are dogs and rodents. Dogs have been found to carry *L. infantum*, *L. tropica*, *L. major* and *L. braziliensis*, while *L. major* is predominantly hosted by gerbils and the fat sand rat (Ashford, 1996). Plantation of new crops is an attractive food source for rodents, increasing the risk of transmission to seasonal workers. Political instabilities, civil unrest and environmental changes (e.g. draughts) are the main causes for migration of human populations. Non-immune individuals from areas where VL is not common are forced to move to areas endemic for leishmaniases, mostly living cramped under bad hygienic conditions in close proximity to parasite reservoirs like dogs. This, for example, led to the spread of VL endemic areas from Sudan to Ethiopia and Eritrea (www.who.int/leishmaniasis/ burden/magnitude/burden_magnitude/en/index.html, 2009; Desjeux, 2001).
A further threat is constituted by the possibility of global warming, moving the maximum northern latitude for sand fly survival further north and increasing the number of countries where VL could become endemic (Desjeux, 2001). Indeed, while endemic in South European countries, sand flies have been found in central European countries like Germany and Belgium since 1999, and are proven or suspected vectors of leishmaniasis (Aspock *et al*., 2008; Naucke *et al*., 2008). In Germany, eleven cases of leishmaniasis, where indigenous origin is likely, were found since 1991, one involving an infant (Naucke *et al*., 2008).

The high incidence of canine leishmaniasis in the Mediterranean area makes dogs the main reservoir for VL in this region. When treated, these dogs receive Miltefosine or AmBisome®, drugs which are used in India to treat human disease. This enhances the risk of the emergence of drug resistant parasites, which then due to globalisation and tourism threaten to spread to developing countries (Dujardin *et al*., 2008).
A further massive problem is the increasing occurrence of VL in patients with HIV. Along with African countries co-infections were also frequently detected in South Europe (Spain, Portugal, France and Italy), where *L. infantum* infection is the third most common parasitic disease in HIV patients (Alvar *et al*., 1997). Between 2 and 9 % of all VL cases are known to be HIV positive, but numbers reach as high as 30 % in endemic areas like Ethiopia.
Co-infection of HIV patients with leishmania parasites is considered a major problem, due to the deadly synergy, both pathogens form. Among others, parasites causing VL have been found to modulate the virus life cycle and to promote viral replication thus resulting in the clinical progression of HIV to AIDS. HIV on the other hand promotes the reactivation of latent leishmania infection, due to a declining immune system and a decrease in pro-inflammatory cytokines IL-12, IFNγ and IL-18 accompanied by an increase in anti-inflammatory cytokines IL-4 and IL-10 (Wolday *et al*., 2000).

Together with high treatment cost of human VL, toxicity of drugs and the emergence of parasite resistance (see section 1.3); the factors described above highlight the need for a vaccine against leishmaniasis.

1.6 Anti-leishmanial vaccines

1.6.1 Requirements

An ideal vaccine against visceral leishmaniasis needs to fulfil a number of requirements. Safety and affordability are top priorities, especially in regard of the main vaccination target, populations in developing countries. It should be capable of initiating a protective $CD4^+$ and $CD8^+$ T cell response, lacking IL-10, and induce a long term immunological memory. Along with prophylactic and therapeutic potential, a broad protection against species causing CL and VL would be preferable. Since

functional cold chains and infrastructure are not very common in developing countries, a stable vaccine formulation which can be stored at room temperature would be of advantage.
The most important requirement of all is feasibility. This is given by the fact that individuals after recovery from infection develop a life long immunity against re-infection.

1.6.2 Leishmanization

To protect individuals from disfiguring facial lesions of CL, virulent parasites from active lesions have been deliberately injected into the skin in non-exposed areas of the body. Lesions healed spontaneously resulting in an acquired T cell mediated immunity against re-infection with CL. This procedure is called leishmanization and has been practiced for over a century. In an approach to cover larger geographical areas cultured parasites were used in the former Soviet Union, Israel and Iran (Greenblatt, 1980). However, the procedure had since been abandoned due to the emergence of safety issues, as for example leishmanization in some cases resulted in the development of large non-healing lesions, which required treatment, exacerbating chronic disease or immunosuppression. Further problems were difficulties in standardisation and loss of parasite virulence after several in vitro passages. Studies carried out in the 1940s using killed parasites alone or with BCG were considered inconclusive and were mainly focussed on CL (reviewed by Khamesipour *et al*., 2006; Kedzierski *et al*., 2006; Coler and Reed, 2005). A further approach currently pursued is the development of live attenuated parasites. It is anticipated that immunisation with genetically engineered parasites which are lacking genes for long term survival *in vivo* or which are equipped with suicidal cassettes can induce a protective immune response, before being cleared. Major drawbacks are the limited protection due to rapid elimination of the parasite by the host and the risk of parasites regaining their ability to cause disease (Khamesipour *et al*., 2006; Kedzierski *et al*., 2006).

1.6.3 Antigens

On the quest of developing vaccines against cutaneous and visceral leishmaniasis a number of parasite antigens have been tested for their potential to induce anti-leishmania immune responses. Leishmania surface protein gp63 and leishmania homologue of receptors for activated C kinase (LACK) have been extensively studied in a wide range of adjuvant and delivery systems. Other antigens include lipophospoglycan (LPG), hydrophilic acylated surface protein B1 (HASPB1), promastigote surface antigen 2 (PSA-2), kinetoplastid membrane protein (KMP-11), thiol-specific-antioxidant (TSA) and the polyantigen Leish-111f, to name a few (summarised by Kedzierski *et al*., 2006; Palatnik-de-Sousa, 2008; Coler and Reed, 2005). In an attempt to tackle leishmania infection at an early stage, salivary proteins of the sand fly have also been evaluated for vaccination. Studies in hamsters and dogs showed promising results with induction of type I responses and significant IFNγ and IL-12 production (Collin *et al*., 2009; Andrade *et al*., 2007).

1.6.4 Adjuvant and delivery systems

Purified antigens on their own are most often not immunogenic and require the addition of adjuvant to induce an immune response. Alum and squalene are the only two adjuvants approved for human vaccines. While they are potent inducers of humoral immunity, their induction of T_H1 responses required for protection against VL is considered to be poor. The combination of IL-12 and antigen does induce a strong T_H1 immune response. However, IL-12 is not recommended for human vaccination as it may promote immune disorders and in addition lacks the ability to induce immunological memory, as seen in mice immunised with LACK (Gurunathan *et al*., 1997; Coler and Reed, 2005). Further approaches tested in animal models so far include DNA vaccination using naked DNA or recombinant Vaccinia virus, which act as adjuvant by activation of the innate immune response via non-methylated CPG sequences and confer protection by a prolonged intracellular expression of vaccination antigen. On top of that production costs are relatively cheap in comparison to purified protein.

Leishmania parasites are intracellular pathogens and like all intracellular pathogens clearance requires a T_H1 response. This led to the exploration of attenuated intracellular pathogens like Bacillus of Calmette and Guerin (BCG), *Salmonella enterica* and *Listeria monocytogenes* as delivery systems for foreign antigens (summarised by Kedzierski *et al*., 2006; Palatnik-de-Sousa, 2008; Coler and Reed, 2005).
A rather exotic and perhaps less feasible approach is the pulsation of DC with antigen or peptide. Although results were promising, large-scale production, provision of infrastructure etc. still needs to be addressed.

Despite all effort, no vaccine against visceral leishmaniasis is available to date. The polyprotein vaccine Leish11f, comprising of antigens TSA, LmSTI1 and LeIF, in combination with adjuvant MPL-SE is the first second generation (i.e. subunit) vaccine where human clinical trial phase 1 and 2 have been completed (Coler *et al*., 2007).

1.7 Attenuated live salmonella as vaccine carriers

The employment of recombinant attenuated *Salmonella enterica* for delivery of foreign antigens in vaccines is a promising alternative to the combination of purified antigen and adjuvant. Low production cost and the possibility of lyophilisation, allowing vaccine storage at room temperature, make this approach an ideal candidate for vaccination in developing countries. Salmonella have been shown to induce a strong mucosal, humoral and cellular immune response in vivo and clearance requires the activation of type I T cells which produce cytokines IFNγ, IL-2 and TNFα. Recent studies have also shown that salmonella are capable of activating cytotoxic $CD8^+$ T cells via MHC-I cross-presentation in humans (Salerno-Goncalves and Sztein, 2009; Winau *et al*., 2004). Since visceral leishmaniasis targets in part the same organs (spleen and liver) as typhoid salmonella, activation of tissue specific cellular responses through vaccination is a desirable feature. In addition, salmonella carry Toll like receptor (TLR) ligands such as lipopolysaccharid (LPS) on their surface, which permits activation of the immune system e.g. via TLR4. This makes the use of potentially toxic adjuvants redundant.

S. typhimurium shows similar effects in mice as *S. typhi* in humans and therefore it can be assumed that findings from mouse vaccination experiments can be translated to human-*S. typhi* interaction. Attenuated *S. typhi* strains Ty21a and CVD908 for example have been approved for human vaccination against typhoid fever. Their efficiency to carry heterologous antigens from pathogens like *Helicobacter pylori*, Hepatitis B virus and *Plasmodium falciparum* have been assessed in different clinical vaccination trials (Galen *et al*., 2009; Aebischer *et al*., 2008). A further advantage of using these strains as vaccine carrier is that the target population will not only be vaccinated against VL but simultaneously against typhoid fever, a co-endemic disease.

Oral administration of attenuated salmonella in general is considered safe and well tolerated. Adverse effects are mild and include gastrointestinal disturbance, headaches or in rarer cases the appearance of rashes. Bacteria are cultured in large-scale bioreactors and the lyophilised product is filled into gelatine capsules, which have been coated with organic solutions to ensure that capsules are dissolved in the gut and not the stomach (Kopecko *et al*., 2009). In the small intestine Salmonella are taken up by M cells and translocated from the intestinal lumen to Peyer's patches, lymph node like structures of the gut, where they reside for several weeks (Dunstan *et al*., 1998). Attached or in intracellular form, they then disseminate via mesenteric lymph nodes to visceral organs like spleen and liver (Mäkelä, 1997). In lymphatic tissue salmonella are taken up by macrophages and dendritic cells, which present epitopes of carrier as well as vaccination antigen via MHC-II molecules and activate specific $CD4^+$ T cells, $CD8^+$ T cells and antibody production.

To test the feasibility of recombinant live salmonella several groups have explored them as carrier organism to protect against different forms of leishmaniasis. In the early 1990s researchers achieved partial protection of mice after prophylactic vaccination with gp63 expressing *S. typhimurium* (Yang *et al*., 1990; Xu *et al*., 1995). Antigen expression in these vaccines was then further improved by the use of inducible promoters (McSorley *et al*., 1997). Gonzalez and colleagues expressed gp63 in a human anti-typhi vaccine candidate CVD908 and tested it in murine models. Vaccinated mice elicited a strong T_H1 response and gp63-specific cytotoxic T cells have been found (Gonzalez *et al*., 1998). Other approaches included the therapeutic

vaccination with salmonella strains individually expressing T_H1 cytokines TNFα, IFNγ, IL-2 and migration inhibitory factor (MIF), the prime-boost combination of a DNA vaccine and recombinant salmonella expressing LACK; both showing promising results (Xu *et al*., 1998; Lange *et al*., 2004). Furthermore it has been shown that the protective effect conferred by salmonella expressing gp63 was dependent on the activation of NRAMP through cascade events triggered by bacterial LPS and the production of IFNγ by NK or T cells. NRAMP activation led to upregulation of TNFα, IL-1β and MHC-II; whereas mutation or polymorphism in the gene resulted in the induction of a T_H2 response along with susceptibility (Soo *et al*., 1998). All these studies indicate that successful vaccination using recombinant salmonella is feasible, but in need of improvement.

1.8 Localisation of antigen in the salmonella carrier

For vaccination using recombinant live salmonella, the topology of antigen delivery plays an important role. Expression of foreign protein on the surface or in the cytoplasm can highly influence the bacterial fitness of the carrier strain, and therewith antigen delivery and induced immune responses. Antigen delivery has been improved by the application of *in vivo* inducible promoters such as P_{pagC}, which allow high-level protein expression *in vivo* but show only low activity *in vitro* (Bumann, 2001). Upon entering the phagosome of APC, where low Mg^{2+}/Ca^{2+} conditions prevail, the salmonella PhoP/PhoQ system becomes activated. Aiming at the rapid induction and expression of immune and environmental modulating proteins, promoters like P_{pagC} become active, not only to express virulence factors but also the protein/antigen of interest. However, dependent on the antigen, its intrinsic toxicity or lability in the cytoplasm can result in insufficient stimulation of the immune system (Hess *et al*., 1996). Alternatively antigens can be expressed on the surface of the salmonella carrier.

Autotransporters have evolved to display on or secrete proteins through the outer membrane of Gram-negative bacteria. The adhesin involved in diffuse adherence (AIDA-I) from enteropathogenic *E. coli* allowed the surface expression of more than 10^5 molecules per cell (Jose, 2006). AIDA fusion constructs consisted of a cholera

toxin signal peptide, targeting a protein of interest for secretion through the inner membrane, followed by this passenger, a linker domain and the actual autotransporter domain. After translation the still cytosolic fusion protein is translocated through the inner membrane to the periplasm. Here the cholera toxin signal peptide is cleaved off and under formation of a β-barrel pore structure the AIDA autotransporter part inserts into the outer membrane. The linker and passenger domains are then transported through the pore and displayed on the surface (Maurer *et al*., 1997).

This system has been exploited by different research groups to express and display proteins and peptides (e.g. heat shock protein Hsp60, cholera toxin B and ureA, a subunit of *Helicobacter pylori* urease) on the surface of Gram-negative bacteria (Kramer *et al*., 2003; Maurer *et al*., 1997; Rizos *et al*., 2003). $CD4^+$ T cells isolated from mice, orally vaccinated with Salmonella expressing an epitope of Hsp60 on the surface via AIDA, did proliferate and produced high amounts of IFNγ in response to antigen-specific re-stimulation (Kramer *et al*., 2003).

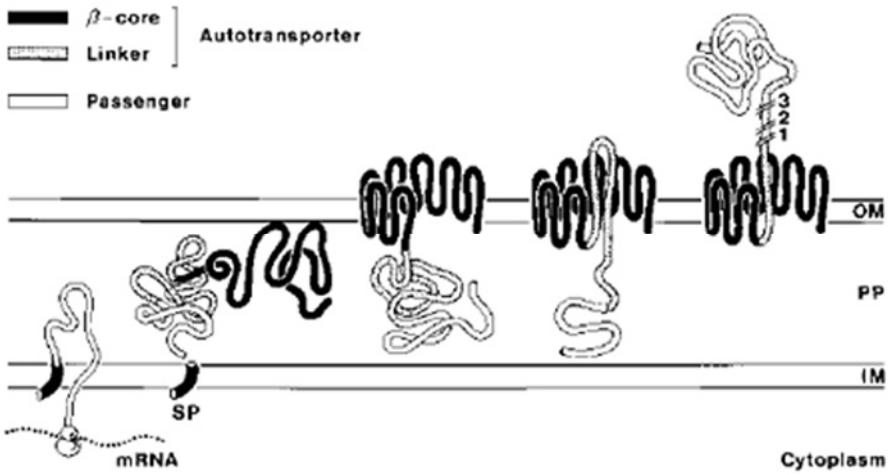

Fig.1.5: Model of antigen exportation via AIDA-I

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1.9 Objectives

The ultimate goal of this project is the pre-clinical development of a recombinant live vaccine against visceral leishmaniasis targeted for the population in India. To reach this goal the following objectives were set:

1. *In silico* selection of novel antigen candidates

Bioinformatic criteria for the selection of vaccine antigens considered of importance for the induction of protective immune responses were to be applied on proteome data sets from *Leishmania mexicana* pro- and amastigotes to identify novel candidate antigens.

2. Construction of optimised live vaccines

Attenuated *Salmonella enterica* serovar Typhimurium SL3261 was chosen as a live carrier to deliver the novel vaccine candidates. Since expression of foreign proteins is known to affect bacterial fitness and therefore the efficiency of a live carrier. The next objective was to optimize these carriers for vaccine delivery by comparing strains engineered to express tuned levels of the novel antigens on the surface or cytosolically.

3. Pre-clinical evaluation of novel live vaccine candidates

A further objective was to obtain proof of concept data that optimized vaccine strains would indeed protect against Leishmaniasis by assessing the novel vaccines in experimental models of leishmaniasis. Emphasis was to be put on analysis of visceralising infection since VL was the target disease. Thus, progressive infection of susceptible BALB/c mice with *L. major* and *L. donovani* respectively were chosen as models.

4. Exploration of outer membrane vesicles (OMV) to augment antigen-specific immune responses

Vaccines often require multiple doses to be effective and prime-boost strategies seem promising against many infections (Prieur *et al*., 2004). Thus, an additional aim was to create a versatile cost effective platform to generate a new kind of booster reagent based on OMVs.

2. Materials and Methods

2.1 Electrical laboratory equipment

ÄKTA prime FPLC system	Amersham pharmacia biotech
Blotting apparatus	Bio-Rad
Centrifuge	Heraeus (Multifuge 3 s-r)
Heat block	Thembloc, Selecta
ELISA plate reader	Multiskan *Ascent*, Labsystems
Electrophoresis system (DNA)	Bio-Rad
Electrophoresis system (Protein)	Bio-Rad
FACS	BD Bioscience (LSRII)
Confocal microscope	Leica SP5
Incubator (with CO_2)	STS (Galaxy S)
Incubator (w/o CO_2)	Heraeus
Incubator, shaking	New Brunswick
Magnetic stirrer	Fisher Scientific
Microscope (inverted)	Olympus CKX41
Microwave	Panasonic
Nanodrop reader	ThermoScientific
PCR machine	Bio-Rad (DNAEngine)
pH meter	WTW (Inolab)
Photo development machine	X-ograph Compact X2
Pipettes (P2, P20, P200, P1000)	Gilson
Pipettes (Multichannel)	Eppendorf
Pipettes (Multidispenser)	Eppendorf
Pipetting aid	Integra Bioscience
Rolling shaker	Hecht assistant
Rotating shaker	Labnet (GyroTwister)
Sonicating water bath	Bandelin (Sonorex)
Spectrophotometer	Amersham Bioscience
Spectrophotometer UV	Eppendorf
Sterile hood	NUAIRE
Table top centrifuge	Eppendorf (5415C)
Ultracentrifuge	Beckman
Ultrasound	Soniprep 150 MSE
Vortex	IKA (MS2 Minishaker)
Water bath	GFL

2.2 Chemicals and reagents

All chemicals have been obtained from Sigma, Fluka, Roth or Riedel-de Haën.

Antibiotics

Ampicillin	stock solution 100 mg/ml in ddH_20
Streptomycin	stock solution 90 mg/ml in ddH_20
Kanamycin	stock solution 50 mg/ml in ddH_20
Gentamycin	stock solution 50 mg/ml in ddH_20
Hygromycin	stock solution 20 mg/ml in EtOH
Pen/Strep	aliquoted stock solution

Antibodies

goat polyclonal anti-mouse IgG2a – HRP conjugated	(Novus Biologicals)
goat polyclonal anti-mouse IgG1 – HRP conjugated	(Novus Biologicals)
donkey anti-mouse IgG (H+L) – HRP conjugated	(Jackson ImmunoResearch)
mouse monoclonal (12CA5) anti-HA – HRP conjugated	(Roche Applied Science)
rat anti-mouse TNF – PE conjugated	(BD Pharmingen)
rat anti-mouse IFNγ – AlexaFluor® 647 conjugated	(BD Pharmingen)
rat anti-mouse IL-4 – AlexaFluor® 488	(BD Pharmingen)
rat anti-mouse CD4 – APC-H7 conjugated	(BD Pharmingen)
rat anti-mouse CD8a – PerCP conjugated	(BD Pharmingen)
anti-mouse CD3 – AlexaFluor® 700	(eBioscience)
rat IgG1 (κ) isotype control – PE conjugated	(BD Pharmingen)
rat IgG1 isotype control – AlexaFluor® 647	(eBioscience)
rat IgG2a isotype control – AlexaFluor® 488	(eBioscience)
mouse anti-08.1140 polyclonal antiserum	(this work)
donkey anti-mouse IgG – Cy™2	(Jackson ImmunoResearch)

Enzymes

Alkaline Phosphatase:	New England Biolabs
Benzonase® Nuclease:	Novagen
T4 Quick ligase	New England Biolabs
Lysozyme	Sigma

Restriction enzymes *Bgl*II, *Bam*HI, *Hind*III, *Nde*I, *Nhe*I, *Sal*I, *Spe*I, *Xba*I, *Xho*I and *Xma*I were all obtained from New England Biolabs.

2.3 Media

2x SDM (semi defined media) for 10 L

Minimum essential medium powder (S-MEM) with Earle's salts, L-glutamine, w/o sodium bicarbonate	140 g
M199 medium powder with Hank' solution, L-glutamine, w/o sodium bicarbonate	40 g
MEM essential amino acids	160 ml
MEM non-essential amino acids	120 ml
Glucose	20 g
HEPES buffer	160 g
MOPS buffer	100 g
Sodiumpyruvate	2 g
L-Alanine	4 g
L-Glutamine	6 g
L-Arginine	2 g
L-Methionine	1.4 g
L-Phenylalanine	1.6 g
L-Proline	12 g
L-Serine	1.2 g
L-Taurine	3.2 g
L-Threonine	7 g
L-Tyrosine	2 g
Adenosine	0.2 g
Guanosine	0.2 g
Glucosamine – HCl	1 g
Folate	0.08 g
p-Aminobenzoic acid	0.04 g
Biotin	0.004 g

Weigh components and fill up with ddH_2O to 9.5 L. Adjust pH to 7.0 with NaOH, add 40 g $NaHCO_3$, readjust pH to 7.3 and fill up to 10 L with ddH_2O. Sterile filter solution and store at -80 °C.

1x SDM for promastigotes

50 % (v/v)	2x SDM
7 % (v/v)	heat inactivated FCS
1 % (v/v)	Pen/Strep solution
42 % (v/v)	sterile ddH_2O

LB-medium

10 g	Tryptone/Peptone
5 g	Yeast extract
10 g	NaCl

Fill up to 1 L with ddH_2O and autoclave.

LB-agar

10 g	Tryptone/Peptone
5 g	Yeast extract
10 g	NaCl
15 g	Agar-Agar

Weigh chemicals into a beaker and add 900 ml ddH_2O. Adjust pH to 7.0 under stirring, fill up to 1 L with ddH_2O and autoclave.

5x M9 salt solution

64 g	Na_2HPO_4
15 g	KH_2PO_4
2.5 g	NaCl
5.0 g	NH_4Cl

Weigh chemicals into a beaker and add 900 ml ddH_2O. Adjust pH to 7.2 under stirring, fill up to 1 L with ddH_2O and autoclave.

M9 minimal medium

200 ml	M9 salts solution
1 ml	10mM $MgSO_4$
20 ml	20% Glucose
10 ml	0.4% Histidine
50 ml	10% Caseine
2 ml	2,3 Dihydroxybenzoic acid (100mg in 1ml ddH_2O and 1ml EtOH)

Fill up to 1 L with ddH_2O, filter sterile and keep at 4 °C.

2.4 Buffers and solutions

10x phosphate buffered saline (PBS)

80 g	NaCl
2 g	KCl
2.4 g	KH_2PO_4
11.5 g	Na_2HPO_4

Dissolve chemicals, adjust pH to 7.2, and fill up to 1 L with ddH_2O, autoclave.

DNA electrophoresis

10x Tris borate EDTA (TBE)

108 g	Tris
55 g	Boric acid
40 ml	0.5 M EDTA (pH 8.0)

Weigh chemicals and fill up to 1 L with ddH_2O, autoclave. For electrophoresis dilute 1:20 in ddH_2O.

6 x DNA loading buffer

0.09 % (w/v)	Bromophenolblue
0.09 % (w/v)	Xylen Cyanol FT
60 % (v/v0	Glycerol
60 mM	EDTA

SDS-PAGE and Western blot

10x SDS running buffer (pH 8.8)

30 g	Tris,
144 g	Glycine
5 g	SDS

Ad 1 L with ddH_2O and adjust pH to 8.8 using HCl.

Coomassie staining solution

0.25 % (w/v)	Coomassie Brilliant-Blue R-250
45 % (v/v)	Methanol
45 % (v/v)	ddH_2O
10 % (v/v)	Glacier acid

Coomassie destaining solution

7 % (v/v)	Glacier acid
20 % (v/v)	Methanol
73 % (v/v)	ddH_2O

SDS-PAGE gel drying solution

35 % (v/v)	Ethanol
2 % (v/v)	Glycerol
63 % (v/v)	ddH_2O

4x protein sample buffer

8 ml	1M Tris/HCl, pH6.8
4 ml	2-Mercaptoethanol
16 ml	Glycerol
12 ml	20% (w/v) SDS
8 mg	Bromophenolblue

Add 2-Mercaptoethanol prior use.

4x SDS-PAGE upper gel buffer (pH 6.8)

30.3 g	Tris
10 ml	10 % (w/v) SDS

Dissolve in ddH_2O, adjust pH to 6.8 using concentrated HCl and fill up to 1 L with ddH_2O.

SDS-PAGE lower gel buffer (pH 8.8)

90. 10 ml 9 g	Tris
	10 % (w/v) SDS

Dissolve in ddH_2O, adjust pH to 8.8 using concentrated HCl and fill up to 1 L with ddH_2O.

10x Transfer buffer (for wetblots)

60 g	Tris
288 g	Glycin

Fill up to 2 L with ddH_2O.

1x Transfer buffer (for wetblots)

100 ml	10x Transfer buffer (see recipe above)
200 ml	Methanol

Fill up to 1 L with ddH_2O.

Amidoblack protein quantification

Amidoblack staining solution

0.5 % (v/v)	Amidoblack
45 % (v/v)	Methanol
45 % (v/v)	ddH_2O
10 % (v/v)	Glacier acid

Protect from light and store at 4 °C.

Amidoblack destaining solution

47.5 % (v/v)	Methanol
47.5 % (v/v)	ddH_2O
5 % (v/v)	Glacier acid

Store at 4 °C.

Amidoblack dissolving solution

80 % (v/v)	Formic Acid
10 % (v/v)	Glacier acid
10 % (w/v)	Trichloroacetic acid

ELISA

10x tris buffered saline (TBS)

24.22 g	Tris
81.81 g	NaCl

Dissolve chemicals in ddH_2O, adjust pH to 7.5 using concentrated HCl and fill up to 1 L with ddH_2O.

Blocking solution

5 % (w/v)	skimmed milk powder in 1 x TBS

Substrate buffer

8.2 g	sodium acetate

ad 1 L with ddH_2O, pH to 5.0 with citric acid

o-Phenylene diamine

10 mg/ml	o-Phenylene diamine dissolved in Methanol

Substrate solution

49.50 ml	substrate buffer
0.5 ml	o-Phenylene diamine
15 µl	30% H_2O_2

Wash buffer

0.05 %	Tween 20 in 1 x TBS

Flow cytometry

FACS-PBS

0.1 % (w/v)	NaN3
0.1 % (w/v)	BSA

in 1xPBS

FACS-PBS-saponin

0.2 % (w/v)	saponin (Sigma)

in FACS-PBS (recipe above)

Protein purification

Lysis buffer (pH 7.5 – 8.0)

25 mM	HEPES
150 mM	NaCl
0.1 % (v/v)	Triton X-100
1 mM	2-Mercaptoethanol
5 mM	Imidazole

Buffer A (pH 7.5)

25 mM	HEPES
150 mM	NaCl

Buffer B (pH 7.5)

25 mM	HEPES
150 mM	NaCl
1 M	Imidazole

All buffers are degassed by sterile filtration.

Resuspension buffer (pH 8.0)

20 mM	Tris-HCl

Isolation buffer (pH 8.0)

2 M	Urea
20 mM	Tris-HCl
0.5 M	NaCl
2 % (v/v)	Triton X-100

Binding buffer (pH 8.0)

6 M	Guanidine hydrochloride
20 mM	Tris-HCl
0.5 M	NaCl
5 mM	Imidazole
1 mM	2-Mercaptoethanol

Wash buffer (pH 8.0)

6 M	Urea
20 mM	Tris-HCl
0.5 M	NaCl
20 mM	Imidazole
1 mM	2-Mercaptoethanol

Refolding buffer (pH 8.0)

20 mM	Tris-HCl
0.5 M	NaCl
20 mM	Imidazole
1 mM	2-Mercaptoethanol

Elution buffer (pH 8.0)

20 mM	Tris-HCl
0.5 M	NaCl
0. 1 mM 5 M	Imidazole (can be increased to 1 M)
	2-Mercaptoethanol

Confocal microscopy

Permeabilisation buffer (in PBS)

0.05 % (w/v)	Saponin
0.1 % (w/v)	NaN_3
2 % (v/v)	FCS
0.1 % (w/v)	BSA

Blocking buffer (in PBS)

3 % (w/v)	BSA
0.1 % (w/v)	NaN_3

2.5 Biologicals

Parasites and isolates

Leishmania major 173::DsRed K2 (Sorensen *et al* 2003)

Leishmania donovani isolate MHOM/INI03BHU-55

Bacterial strains

SL3261	*Salmonella typhimurim*, Δ*aro*A, Strep^R Source: Stocker (Hoiseth and Stocker, 1981)
Escherichia coli JK231	*azi*-6 *fhu*A23 *lac*Y1 *leu-6 mtl-1 pro*C14 *pur*E42 *rps*L109 *thi-1 trp*E38 *tsx-67* Δ(*omp*T-*fep*) *zi*h::T*n10* *dsb*A::Kan Source: Jose (Jose *et al*., 1996)
Escherichia coli BL21 CodonPlus(DE3)-RIPL	F– *ompT hsdS*(rB– mB–) *dcm*+ Tetr *gal* λ(DE3) *endA* Hte [*argU proL* Camr] [*argU ileY leuW* Strep/Specr] Source: Stratagene
Escherichia coli DH5α	Φ80, *lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsdR*17, *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*) *U*169 Source: Promega

Primer

Table 2.1: List of primer used in this study

Name	Sequence 5'-3'	Project/ Specification	Source
LS_KRI XhoI (F)	GATCAA**CTCGAG**GACGTCGGCAAATTATATCGGTCGCGC	Cloning of pAIDA0	This work
LS_SpeI/BglII (R)	GATCAA**AGATCT**TACTAA**ACTAGT**AGGTGTTCCGTGTGCATATGC	Cloning of pAIDA0	This work
KOR_RBS6 (F)	GATCAA**TCTAGA**TTTAAGAAAGAAATATACATATGATTAAATTAAAATTTGGTG	RBS mutation	Konstanin Rizos
KOR_RBS4 (F)	GATCAA**TCTAGA**TTTAAGAAGCAGATATACATATGATTAAATTAAAATTTGGTG	RBS mutation	Konstanin Rizos
AIDA RBS_2 (F)	GATCAA**TCTAGA**TTTAAGAAGGGAATATACATATGATTAAATTAAAATTTGGTG	RBS mutation	This work
KMP_11 SpeI (F)	GATCAA**ACTAGT**GCCACCACGTACGAGGAG	Surface expression	This work
KMP_11 L.inf (R)	GATCAA**AGATCT**CTTGGATGGGTACTGCGCAGCC	Surface expression	This work
223_KMP_NdeI (F)	GATCAA**CATATG**GCCACCACGTACGAGGAG	Cytosol expression	This work
223_KMP_BamHI (R)	GATCAA**GGATCC**GTCGATTACTTGGATGGGTACTGCGCAGCC	Cytosol expression	This work
Lin09.1180 NdeI (F)	GATCAA**CATATG**TCCCCTCTGCAGCAGGCACGCTGG	Cytosol expression	This work
Lin09.1180 BamHI (R)	GATCAA**GGATCC**GTCGACTACACTTTTCGGGAAAAACC	Cytosol expression	This work
Lin09.1180 SpeI (F)	GATCAA**ACTAGT**TCCCCTCTGCAGCAGGCACGCTGG	Surface expression	This work
Lin09.1180 BglII (R)	GATCAA**AGATCT**CACTTTTCGGGAAAAACCAGTG	Surface expression	This work
Lin23.0410 NdeI (F)	GATCAA**CATATG**CTTCACTTCCCCATTTCGCCC	Cytosol expression	This work
Lin23.0410 BamHI (R)	GATCAA**GGATCC**GTCGATTACCAGCCGCGGTGATAGAGG	Cytosol expression	This work
Lin23.0410 SpeI (F)	GATCAA**ACTAGT**CTTCACTTCCCCATTTCGCCC	Surface expression	This work
Lin23.0410 BglII (R)	GATCAA**AGATCT**CCAGCCGCGGTGATAGAGG	Surface expression	This work
Lin25.1680 NdeI (F)	GATCAA**CATATG**TCGTCCGAGGTTGCGATTCAGC	Cytosol expression	This work
Lin25.1680 BamHI (R)	GATCAA**GGATCC**GTCGACTACTGCTGCTGCTTCTCC	Cytosol expression	This work

Name	Sequence 5'-3'	Project/ Specification	Source
Lin25.1680 SpeI (F)	GATCAA**ACTAGT**TCGTCCGAGGTTGCGATTCAGC	Surface expression	This work
Lin25.1680 BglII (R)	GATCAA**AGATCT**CTGCTGCTGCTTCTCCGG	Surface expression	This work
Lin35.0240 NdeI (F)	GATCAA**CATATG**CTGCGCCACTCGCTGCTTCG	Cytosol expression	This work
Lin35.0240 BamHI (R)	GATCAA**GGATCC**GTCGACTACCACCAGGCTGCCTTGC	Cytosol expression	This work
LinJ35.0240 SpeI (F)	GATCAA**ACTAGT**CTGCGCCACTCGCTGCTTCG	Surface expression	This work
Lin35.0240 BgIII (R)	GATCAA**AGATCT**CCACCAGGCTGCCTTGCGGATGC	Surface expression	This work
Lin08.1140 NdeI (F)	GATCAA**CATATG**TCTCAGCAGCTCGCCTTCC	Cytosol expression	This work
Lin08.1140 BamHI (R)	GATCAA**GGATCC**GTCGATTACGGGTGGCTGTCGTCG	Cytosol expression	This work
Lin08.1140 SpeI (F)	GATCAA**ACTAGT**TCTCAGCAGCTCGCCTTCC	Surface expression	This work
Lin08.1140 BglII (R)	GATCAA**AGATCT**CGGGTGGCTGTCGTCGGCGGC	Surface expression	This work
cVAC-seq (F)	GCAGGTGTTTAAACACACCG	Sequencing (pcVAC)	This work
cVAC-seq (R)	GGTTATGCTAGTTATTGCTC	Sequencing (pcVAC)	This work
sVAC-seq (F)	GCATAGTGGCGATAACTGAATGCC	Sequencing (psVAC)	This work
sVAC-seq (R)	CACGGTAAGAGTATTACCTGC	Sequencing (psVAC)	This work
T7 Promoter	TAATACGACTCACTATAGGG	Sequencing (pET28a)	Novagen
T7 Terminator rev	TATGCTAGTTATTGCTCAG	Sequencing (pET28a)	Novagen
pPro18-NheI Forw	GATCAA**GCTAGC**TTTAAGAAGGAGATATACATATGATTAAATTAAAATTTGGTG	Membrane vesicles	This work
pPro18-SalI Rev	GATCAA**GTCGAC**TCAGAAGCTGTATTTTATCCCCAGTGC	Membrane vesicles	This work
pMV-AIDA seq F	CATTTAATTTTATTAAGGC	Sequencing (pMV1)	This work
pMV-AIDA seq R	CGTTCTGATTTAATCTGTATC	Sequencing (pMV1)	This work

Restriction sites are in bold, mutated ribosomal binding site (RBS) is underlined

2.6 Molecular biological methods

2.6.1 Site-directed mutagenesis and primer design

In order to introduce specific point mutations or restriction sites to a construct a PCR was performed using specifically designed primers (table 2.1). Amplification was carried out using the Platinum® *Pfx* DNA polymerase kit (Invitrogen) following manufacturers instructions.

Template:	0.5 – 1 µl
10x Pfx Amplifiaction buffer:	10 µl
dNTPs (10 mM):	4 µl
Primer (10 µM):	4 µl each
$MgSO_4$ (50 mM):	2 µl
Platinum® Pfx DNA Polymerase:	1 µl
ddH_2O:	ad 100 µl

The reaction was started with incubation for 5 min at 94 °C. This was followed by 35 cycles of denaturing 15 s at 94 °C, annealing 30 s at 55 °C and extension 60 s at 68 °C. The final step was a 7 min extension at 68 °C.
Gene specific primers were designed using sequence information from GeneDB. At least half of the primer sequence was matched with the gene, with at least two guanines or cytosines at the 3' terminus to favour stable annealing. A 5' restriction site was introduced to allow subsequent cloning of the PCR product.

2.6.2 Preparation of DNA for Ligation

DNA was digested using 1.5 µl of appropriate restriction enzymes (New England Biolabs) in buffer provided with the enzymes in a final volume of 50 µl. Alkaline phosphatase (New England Biolabs) was added to the plasmid digest in order to remove phosphate residues. All digests were incubated for 1 h at 37 °C. For further processing, enzymes were either removed by agarose gel electrophoresis or on column using the QIAquick PCR Purification Kit (Qiagen).

2.6.3 PCR purification

To remove enzymes off nucleotides after PCR or restriction digestion 5 volumes of buffer PBI was added to 1 volume of reaction. The purification was carried out using the QIAquick PCR Purification Kit (Qiagen) following the manufacturers instructions.

2.6.4 DNA sequencing

Samples were sequenced by the internal SBS Sequencing Service, Ashworth Laboratories of the University of Edinburgh. Results were analysed using Chromas MFC software and NCBI Blast.

2.6.5 Ligation

For ligation the Quick Ligation Kit (New England Biolabs) was used. Briefly, different ratios of plasmid and insert were mixed in 9 µl H_2O with 10 µl 2x ligation buffer and 1 µl of Quick T4 DNA ligase. Vector self-ligation control was performed by replacing the insert with the same volume of water. The reaction was left at room temperature for not longer than 10 min and half of the reaction was used for subsequent transformation. The rest was frozen at -20 °C to permit another transformation.

2.6.6 Preparation of chemically competent cells

Two hundred ml of LB media containing the appropriate antibiotics were inoculated 1:50 with an overnight preculture. The cells were grown at 37 °C for about 3 h till an OD_{600nm} of 0.3 was reached. After centrifugation for 10 min at 3000 xg and 4 °C in four 50 ml tubes the supernatant was discarded and the pellets were resuspended in 8 ml of ice cold 100 mM calcium chloride combining cells from two tubes. The cells

were incubated for at least 20 min on ice before centrifuged again as above. The combined pellets were resuspended in 8 ml of ice cold calcium chloride and left of ice over night at 4 °C. The following day 4 ml of ice cold 60 % glycerol were added and cells were frozen in aliquots at -80 °C.

2.6.7 Transformation

Two hundred µl of competent cells were added to either 1-10 µl of plasmid or 10 µl of ligated DNA. The cells were left on ice for 10 min., then heat shocked at 42 °C for 90 s and were immediately placed on ice for further 2 min. Eight hundred µl of LB medium were added and cells were allowed to grow at 37 °C for at least 1 h. Then cells were pelleted for 1 min at 13200 rpm in a table top centrifuge and 800 µl of the supernatant was removed. The pellet was resuspended in the remaining 200 µl and plated on LB agar containing the appropriate antibiotics.

2.6.8 Agarose gel electrophoresis

Agarose was weighed and boiled in 0.5 % TBE according to the required concentration (0.8 to 1.5 %). The gel chamber and the combs were assembled. After cooling to ~55 °C ethidium bromide (to 0.001 %) was added and the gels (60 ml for a small gel, 120 ml for a large gel) were cast. Once the gels settled they were placed in an electrophoresis chamber containing 0.5 % TBE. Samples and marker were loaded and the gels were run at 95 V (small gel) or 120 V (large gel) for the time required.

2.6.9 DNA extraction from gels

After running the DNA samples on agarose gels, fragments of interest were isolated from the gel using a clean scalpel and the DNA was extracted using the QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions.

2.6.10 Colony PCR

For quick screening of recombinant bacteria, single colonies were picked and resuspended in 50 µl PBS. Subsequently the tip with the remaining bacteria was streaked on an LB agar plate and incubated at 37 °C over night. From the suspension 1 µl was pipetted into a PCR tube and the following reagents were added: dNTP-mix (0.4 mM), forward and reverse primer (each 100 µM), 10 µl 5x Mango Taq Coloured Reaction Buffer (Bioline), $MgCl_2$ (2 mM) and 1.25 U Mango Taq DNA polymerase (Bioline). Water was added to make 50 µl of total volume.
The reaction was started with incubation for 5 min at 94 °C. This was followed by 30 cycles of denaturing 1 min at 94 °C, annealing 2 min at 54 °C and extension 3 min at 72 °C. The final step was a 7 min extension at 72 °C.

2.7 Protein techniques

2.7.1 Amidoblack protein quantification

This method was used for samples which contain reagents (e.g. SDS, 2-Mercaptoethanol etc) which are known to interfere with normal protein quantification methods (e.g. Lowry, Bradford etc.) (Dieckmann-Schuppert and Schnittler, 1997).
A cellulose acetate membrane was divided in 1.25 cm x 1.25 cm squares, fixed with two clips and hang over a tray. Samples were prepared by boiling equal volumes of sample and 2x protein sample buffer for 5 min. A BSA standard dilution series (0, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5 and 5 mg/ml) in PBS was prepared the same way and after boiling and a quick spin 5 µl of sample per square were applied. The membrane was dried for 10 min and then covered in Amidoblack staining solution for 10 min under shaking. The staining solution was then discarded; the membrane rinsed with water and then destained 3x 5 min. The cellulose acetate sheet was allowed to dry thoroughly before the squares were cut and placed into 1.5 ml reaction vessels. 500 µl of dissolving solution was added and the samples were incubated for 30 min at 50 °C under shaking. After the dissolving process, 250 µl of samples were pipetted in a 96-well microtitre plate and the absorbance was measured at 620 nm (or the nearest wavelength available). From the single values the value of a

water control (0 mg/ml) was subtracted and subsequently the protein concentration determined based on a standard curve.

2.7.2 SDS-PAGE

Glass plates were cleaned and fitted into the casting stand. The resolving gel (10 %, 12 % or 15 %) was prepared (see table 2.2) and poured to approximately 1.5 cm below the top, and covered with 100 % isopropanol. After polymerisation the isopropanol layer was removed and the stacking gel was prepared and poured on top of the resolving gel. A comb was placed in the still liquid stacking gel and the gels were allowed to polymerise. Once settled the gels were placed into the tank and 1x SDS running buffer was added. Samples were mixed in protein sample buffer and together with the protein marker were boiled for 5 min and loaded onto the gel.

Table 2.2: Pipetting scheme for SDS-Gels of different percentage

Resolving gel	10 %		12 %		15 %	
	1 gel	2 gels	1 gel	2 gels	1 gel	2 gels
H_2O	2.5 ml	5.0 ml	2.1 ml	4.2 ml	1.5 ml	3.0 ml
4x lower gel buffer	1.5 ml	3.0 ml	1.5 ml	3.0 ml	1.5 ml	3.0 ml
40 % acrylamide	2.0 ml	4.0 ml	2.4 ml	4.8 ml	3.0 ml	6.0 ml
TEMED	4.5 µl	9.0 µl	4.5 µl	9.0 µl	4.5 µl	9.0 µl
10 % APS	45 µl	90 µl	45 µl	90 µl	45 µl	90 µl
total volume	6 ml	12 ml	6 ml	12 ml	6 ml	12 ml

Stacking gel	5 %	
	1 gel	2 gels
H_2O	1.17 ml	2.34 ml
4x upper gel buffer	0.5 ml	1.0 ml
40 % acrylamide	0.33 ml	0.66 ml
TEMED	3.0 µl	6.0 µl
10 % APS	25 µl	50 µl
total volume	2 ml	4 ml

For focussing gels were run at 70 V till the samples reached the resolving gel. Then voltage was raised to 115 V and the samples were run till the bromophenolblue dye reached (or ran out of) the bottom of the gel. Gels were removed from the glass plates and stained for 15-20 min in Coomassie staining solution (Laemmli, 1970). Afterwards gels were destained and dried between cellulose sheets.

2.7.3 Western blot analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis of gels of appropriate percentage. After electrophoresis, gels were soaked in 1x transfer buffer for 5 min. Using a wet blot system (BioRad) two pieces of pre-cut filter paper and two fibre pads were soaked in transferbuffer. A 9 x 6 cm PVDF membrane (Millipore) was cut and briefly rinsed in methanol. After another rinse in water, the membrane was placed in transfer buffer as well. To assemble the transfer sandwich, one fibre pad was put on one site of the blotting cassette followed by a filter paper. The equilibrated gel was placed onto the filter paper and trapped air bubbles were removed. Then the membrane was laid on top of the gel and the sandwich was completed by another filter paper and fibre pad. Air bubbles were removed with help of a pen. The cassette was closed and then placed in the module and the buffer tank. The ice unit was added and the tank was filled up with transfer buffer. After one hour of transfer at 100 V under permanent stirring the sandwich was removed, the membrane was placed in a 50 ml tube and blocked with PBS/ 5 % skimmed milk powder for 15 min. Antibody was pre-incubated in PBS/ 5 % milk (and *Salmonella typhimurium* lysate when required) for 30 min. Afterwards the membrane was incubated for 1 h with the antibody solution on a rolling shaker. When incubation with a secondary antibody was necessary, the membrane was washed for 3x 10 min with PBS/ Tween 20 between both incubation periods. Before detection the membrane was washed 3x 10 min to remove unbound antibodies. Membranes were then placed in a cassette and detection was performed using the ECL kit (Pierce) following manufacturers instructions.

2.8 Protein purification

2.8.1 Induction of recombinant protein

A single colony was picked and used to inoculate 10 ml of LB medium supplemented with 50 µg/ ml kanamycin and grown overnight. Five hundred ml of LB medium was inoculated with 0.5 ml of an over night culture and was grown at 37 °C, 150 rpm till an OD_{600nm} between 0.4 and 0.6 was reached. At this stage 1 ml culture sample was removed, bacteria centrifuged at 20800 xg for 3 min; the resulting pellet was resuspended in 50 µl 2x protein sample buffer and boiled for 5 min at 95 °C and frozen at -20 °C till later analysis. Recombinant protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, 0.4 – 0.8 mM) to the cultures which were then incubated at 30 °C for 5 h with shaking. After induction OD_{600nm} was measured and 0.5 ml of culture was removed for an analytical SDS-gel. Finally, all bacteria were harvested by centrifugation for 25 min at 4500 xg and 4 °C. The supernatant was discarded; the pellet was washed once with 30 ml lysis buffer and was then transferred into a 50 ml tube. After another spin for 25 min at 4500 xg and 4 °C the pellets were stored at -80 °C until further processing.

2.8.2 Purification of soluble proteins

The bacterial pellets were thawed and resuspended in 5 ml lysis buffer supplemented with half a tablet protease inhibitors (Complete Mini EDTA-free tablets, Roche) and 1 mg/ ml lysozyme (Sigma). Afterwards the suspension was gently shaken at 30 °C in a water bath for 1 h till the solution became viscous. Twenty-five units of DNase (Benzonase®Nuclease, Novagen) were added and the suspension was allowed to rest on ice for further 30 min. The bacteria were then disrupted by sonication on ice (4x 30 s intervals with 30 s resting between) until the solution became clear. After a centrifugation step at 18000 xg and 4 °C for 25 min the supernatant was filtered through a 0.22 µm pore sized filter and then purified by affinity chromatography. Preloaded nickel columns (GE Healthcare) were equilibrated with 5 column volumes (CV) Buffer A at a flow rate of 1 CV/ ml. After sample application columns were washed with 20 CV Buffer A : Buffer B (ratio 99:1). Recombinant proteins were

eluted on a continuous gradient towards a Buffer A/Buffer B ratio of 50:50, during which fractions of 1 CV were collected. Finally the gradient was raised to 100 % Buffer B in order to elute the remaining proteins.

2.8.3 Purification of proteins from inclusion bodies

The bacterial cell paste was thawed and resuspended in 20 ml resuspension buffer supplemented with protease inhibitors (Complete Mini EDTA-free tablets, Roche) and 1 mg/ ml lysozyme (Sigma). Afterwards the suspension was gently shaken at 30 °C in a water bath for 1 h till the solution became viscous. Twenty-five units of DNase (Benzonase®Nuclease, Novagen) were added and the suspension was incubated on ice for further 30 min. The bacteria were then further disrupted by sonication on ice (4x 30 s intervals with 30 s resting between). After a centrifugation step at 25000 xg and 4 °C for 10 min the supernatant was discarded and the pellet was resuspended in 15 ml cold isolation buffer. The sonication was repeated as described above. Afterwards centrifugation, re-suspension and sonication were repeated for a second time. The suspension was pelleted again and the precipitate was resuspended in 10 ml of buffer without urea (e.g. resuspension buffer). After a final spin at the conditions mentioned above the resulting pellets were frozen at -80 °C till further processing.

Frozen pellets were dissolved in 5 ml binding buffer per 100 ml induced culture. Samples were incubated for 30 to 60 min at room temperature with gently shaking. Insoluble parts were removed by centrifugation (25000 xg, 15 min and 4 °C) and the supernatant was passed through a 0.45 µm filter.

For chromatography affinity columns preloaded with nickel were equilibrated with 10 CV of binding buffer at a flow rate of 1 CV/ min. Once no protein or other contaminations were detectable the sample was applied onto the column. Afterwards unbound proteins were washed with 30 CV of wash buffer and the flow through was collected. In order to allow complete refolding of the proteins a gradient (wash buffer to refolding buffer) of 45 CV was applied and flow rate for this step was reduced to 0.5 CV/ min. This was followed by further 10 CV of refolding buffer. Refolded proteins were eluted with 20 CV of a gradient (refolding buffer to elution buffer) during which fractions of 1 CV were collected. Fraction collection was continued

5 CV beyond this gradient. The final step was a wash of the column in 10 CV binding buffer in order to allow complete removal of remaining protein from the column. After the final run, columns were washed with 20 % ethanol and stored at 4 °C.

2.9 Parasite cultures

2.9.1 Maintenance of leishmania promastigote cultures

Leishmania promastigotes were grown in 1x SDM with 20 µg/ ml hygromycin without CO_2 at 28 °C. Once the stationary phase was reached, 100 µl of the old culture was used as inoculum for a new 10 ml promastigote culture. In order to maintain virulence, parasites were regularly passaged through a host. For that purpose mice were infected with 2x 10^6 promastigotes sub-cutaneously at the tail base. After a lesion became visible, mice were sacrificed. The lesion and the draining lymph node were removed and transferred into fresh SDM medium to allow growth of virulent parasites. After one passage in culture, parasites were frozen at -80 °C.

2.9.2 Freeze/thawing of leishmania parasites

For freezing 1x 10^7 parasites of logarithmic promastigotes culture were spun down at 1100 xg at 4 °C for 10 min. The supernatant was discarded and the pellet containing the parasites was restored in 500 µl cold SDM medium. Five hundred µl cold 15 % glycerol solution was added and after mixing, the parasites were transferred into a cryo tube. These were then placed in a cryo box and frozen slowly to -80 °C for 24 h. Subsequently tubes were transferred into liquid nitrogen to allow long term storage.
For thawing parasites were quickly thawed in a 37 °C water bath and then transferred into a tube containing 10 ml SDM medium. After centrifugation for 10 min at 1100 xg and room temperature the resulting pellet was resuspended in 1 ml SDM which was then used to inoculate several flasks containing SDM medium with different volumes of parasites. After roughly four days in culture parasites were passaged as usual.

2.10 Animals, immunisation and protection experiments

2.10.1 Mice

Female BALB/c or C57BL/6 mice were purchased from Harlan UK, Charles River UK or bred at the University animal facilities and maintained under specific pathogen-free conditions in individually ventilated cages. Animals were used at 6–9 weeks of age and were age matched within each experiment. All experiments were approved by a Project License granted by the Home Office (U.K.) and conducted in accordance with local guidelines.

2.10.2 Preparations of frozen salmonella stocks for immunisation

Two hundred ml of LB medium containing the appropriate antibiotics were inoculated 1:100 with a pre-culture and grown at 37 °C; 150 rpm till an OD_{600nm} of 1.4-1.6 was reached. The cultures were centrifuged at 4 °C, 3500xg for 20min and the supernatant was discarded. The pellets were resuspended in 10 ml of LB medium and more medium was added to a volume of 35 ml. Centrifugation was repeated at 4 °C, 3500 xg for 15 min, the supernatant was removed and the pellet was resuspended in 300 µl LB/ 30 % Glycerol. A small sample was diluted to determine OD_{600nm}, the suspension was adjusted to a density equivalent to OD_{600nm} = 100, which corresponds to approximately 10^{10} CFU. Suitable aliquots were frozen away in cryotubes at -80 °C. For exact determination of vaccination doses, an aliquot was thawed on ice, serially diluted and dilutions plated on LB agar plates containing appropriate antibiotics. After growth at 37 °C, colonies were counted and CFU numbers calculated.

2.10.3 Purification of outer membrane vesicles

One litre of LB medium with the appropriate antibiotics was inoculated with 10 ml of a preculture grown from a single colony. When the culture reached an OD_{600nm} between 0.6-0.8, 1 ml of sample was withdrawn and sodium propionate to 50 mM was added to induce the production of AIDA-fusion proteins on the cell surface. Samples were regularly removed to monitor recombinant protein expression and release into the supernatant. These samples were centrifuged for 3 min at 20800 xg, 100 µl of supernatant were taken and the rest discarded. The pellet was dissolved in 50 µl of 2x protein sample buffer and boiled for 5 min at 95 °C. Pellets and supernatant were frozen at 20 °C until further analysis. After approximately 10 h of induction 50 µg/ ml gentamycin was added for 30 min in order to increase membrane shedding. The cultures were then harvested at 4500 xg for 30 min, the pellets discarded and the supernatant was passed through a 22 µm pore size filter unit. This was followed by ammonium sulphate precipitation using 390 g/ L ammonium sulphate to obtain 60 % saturation. The solution was left to settle over night at 4 °C. The next morning the precipitate was harvested by centrifugation for 30 min at 11000 xg at 4 °C. The pellets were dissolved in 20 ml PBS and spun again for 15 min at 16000 xg and 4 °C. Afterwards supernatants were transferred into Beckman ultracentrifugation tubes and membrane vesicles were pelleted for 2 h at 100000 xg and 4 °C. To determine dry weights, pellets were dissolved in endotoxin free water and lyophilised. Vesicles were reconstituted in endotoxin free water (HyCult biotechnology bv) to a working concentration of 1 mg/ ml and stored at 4 °C.

2.10.4 Determination of bacterial fitness by colonisation assay

Three mice per group were immunized orally with 1 x 10^{10} CFU of a SL3261 vaccine. After seven days mice were sacrificed and Peyer's patches removed and placed in a vial containing 1 ml PBS. Subsequently, Peyer's patches were homogenized between the rough ends of two glass slides and the resulting suspension was transferred into a 2 ml reaction tube. The glass slides were rinsed with an additional 1 ml of PBS. 200 µl of the suspension, 100 µl of 1 % Triton X-100 and 700 µl of PBS were mixed and 100 µl were plated on a selective LB agar plate in duplicates. Out of this dilution

two further dilutions (1:10 and 1:100 respectively) were prepared and also plated on LB agar plates. All plates were incubated overnight at 37 °C and colony numbers were counted the following day.

2.10.5 Infection of mice with *L. major* and foot pad measurements

L. major promastigotes were grown in 1 x SDM until stationary phase was reached. Parasites were counted and adjusted to 1 x 10^7/ 100 µl in PBS and 20 µl (equal 2 x 10^6 parasites) were injected into the left hind foot pad. Foot pad swelling was measured using a calliper. To determine the overall swelling, the difference in thickness between left infected foot pad and right reference foot pad was calculated.

2.10.6 Determination of *L. major* burden in murine organs

Mice were sacrificed by cerebral dislocation; organs (spleen, liver, draining lymph node, foot pad) were removed and placed into tubes containing 1 ml of PBS. For processing, organs were forced through a cell strainer using the sterile top end of a 1 ml syringe. Before this treatment, feet were cut several times with scissors to simplify homogenisation. Cell strainers were flushed with 1 ml of PBS and the single cell/ parasite suspension was pipetted back into the collection tube. After homogenisation volumes in all tubes were adjusted to the same level.

For the serial dilution assay, sterile 96 well plates were labelled and filled with 100 µl 1x SDM (supplemented with 20 µg/ ml hygromycin and 50 µg/ ml kanamycin) per well. Homogenates of highly infected foot pads and lymph nodes were pre-diluted 1:100. For serial dilution, 46 µl of cell suspension were added into the wells of the first column in quadruplicates and 46 µl were serially transferred resulting in a 1:√10 dilution steps over all 12 wells of a row. The plates were finally sealed with parafilm and incubated at 27 °C for 14 days. Parasite growth was then scored microscopically and parasite load in the infected organs was calculated using the dilution where at least 2 of 4 wells (~37.5 %) of wells were positive (Taswell *et al*., 1980). The

reciprocal of this dilution was multiplied by the total volume (in multiples of 0.1 ml) to derive the total number of parasites per organ.

2.10.7 Determination of hepatosplenomegally and *L. donovani* burden in impression smears

Mice were sacrificed, weighed, liver and spleen removed and the weight of both organs was also determined. The organ-body-mass index (BMI) was calculated as the percentage of weight of the single organ to the total body weight of the mouse. To determine parasitic burden in spleen and liver, impression smears from cut organs were prepared on microscopic glass slides. Slides were fixed in methanol and stained with Giemsa. Afterwards the number of parasites per 1000 host cell nuclei was counted using a bright-field microscope and an immersion oil lens. Leishman-Donovan units (LDU) were calculated by multiplying the number of parasites/ 1000 nuclei by the organ weight.

2.10.8 Giemsa staining

Impression smears were briefly fixed in a small volume of methanol. After pouring off excess methanol slides were air dried. Giemsa reagent was prepared in a 50 ml tube by mixing 3.6 ml Sorenson A (9.5g Na_2HPO_4/L ddH_2O), 1.4 ml Sorenson B (9.07g KH_2PO_4/L dH_2O), 45 ml ddH_2O and 5.55 ml of a 1:10 dilution of Giemsa stain. The staining solution was mixed carefully and poured into staining baths. Slides were placed upside down in the staining bath and left for 20 to 30 min at room temperature. The stain was then rinsed off under running tap water and slides were allowed to dry.

2.10.9 Blood collection and serum preparation

Mice were bled by submandibular stab incision in accordance to Home Office guidelines. Blood was collected in a 1.5 ml tube and left to clot at 4 °C over night.

The following day, clot and serum were separated by centrifugation at 20800 xg for 15 min and serum was carefully removed and transferred to a new tube, frozen and stored at -80 °C till further use.

2.11 Immunological methods

2.11.1 ELISA

96 well plates were coated overnight with 50 µl recombinant antigen (50 µg/ml) or 5-7.5x 10^7 bacterial equivalents of *Salmonella enterica* serovar Typhimurium-OH test antigen (TS1611, Sifin) per well. The next day plates were tapped dry and washed with PBS/0.05 % Tween-20 for 5 min. Subsequently, free binding sites on plates were blocked with 80 µl/ well skimmed milk/TBS for 45 min at 28 °C (alternatively 30 min at 37 °C). Master serum dilution plates were prepared by pipetting 2.5 % skimmed milk/TBS to each well, adding 40 µl of pooled sera of each vaccination group in triplicates to the top row and serially diluted to 1:5; 1:20; 1:80; 1:320; 1:1280; 1:5120; 1:20480; 1:81920. When obtained serum volumes were sufficient, sera from single mice were diluted individually omitting technical replicates.
Blocked, antigen coated plates were washed three times with PBS/0.05 % Tween-20 and 50 µl of the serum dilutions were transferred from the master plates to antigen coated plates. After incubation for 2 h at 37 °C plates were washed three times and 50 µl of diluted horseradish peroxidase-conjugated detection antibodies (goat anti-mouse IgG1 or IgG2a, Novus Biologicals, 1:10000 diluted; donkey anti-mouse IgG, Jackson ImmunoResearch, 1:2500 diluted) were added to each well. Plates were incubated for a further hour at 37 °C. This was followed by another wash and 80 µl of substrate buffer was added to each well for 5 min. Plates were tapped dry and 50 µl of substrate solution was pipetted into each well. After incubation for 30 min at 37 °C reactions were stopped with 10 µl 5 M H_2SO_4. The absorption was read at 490 nm using an ELISA plate reader. For analysis, triplicate values were averaged and the absorption at 490 nm against the dilution was plotted. Antibody titres were determined by reading the value at half-maximal absorption. Any curves which did not reach the half-maximum were classified as below detection limit.

2.11.2 Flow cytometry (FACS)

Single cell suspensions from murine spleens were prepared and 1-3x10^7 viable cells were re-stimulated either with 5-20 µg purified antigen (for 18 h), with medium alone (for 18 h) or with PMA/Ionomycin (for 6 h). Two hours before harvest, Brefeldin A (10 µg/ml) was added to allow accumulation of intracellular cytokines.
Cells were then harvested by centrifugation (350 xg, 5 min), resuspended in FACS-PBS and transferred into FACS tubes. For surface staining (CD3, CD4 and CD8a) antibody cocktails (0.4 µg of each antibody per sample) were prepared in FACS-PBS and added to isotype and ICS samples. Cells were incubated for 30 min at 4 °C in the dark and then washed once with FACS-PBS prior fixation with Reagent A (Caltag) for 15 min at room temperature. Afterwards cells were again washed in FACS-PBS and fixative was removed by centrifugation. All subsequent steps were performed in the dark. For intracellular staining (ICS) cells were permeabilised with 100µl Reagent B (Caltag), and antibody cocktails for ICS (α-TNFα-PE, α-IFNγ-Alexa®647 or α-IL-4-Alexa®488) or respective isotype controls were added. After incubation for 45 min at room temperature samples were washed in FACS-PBS-saponin twice and were resuspended in FACS-PBS for immediate analysis or in 0.1 % paraformaldehyde fixative solution then stored at 4 °C in the dark for later analysis.
Data from 2x 10^5 to 1x 10^6 cells were acquired on a LSR II (BD Bioscience) using FACSDiva software (BD Bioscience) and analysed using FlowJo software (Tree Star, Inc).

2.11.3 Staining of leishmania parasites for fluorescent microscopy

Parasites from late logarithmic phase cultures were harvested by centrifugation (5 min, 1900 xg) at room temperature, the supernatant discarded and the pellet washed twice with PBS. The parasites were fixed in 4 % paraformaldehyde/HEPES for 30 min at room temperature. In order to quench free aldehydes, fixed parasites were washed once in TBS, the resulting pellet was resuspended in 100 µl TBS and a droplet each was carefully distributed on Polysine (VWR International) slides and left to dry. For intracellular staining parasites were covered in permeabilisation buffer and incubated in a wet chamber for 10 min at room temperature. Parasites, which were

surface stained only, were treated equally with buffer lacking saponin. Afterwards slides were washed once with PBS and blocked with buffer containing 3 % BSA for 1 h in a wet chamber, then washed again with PBS. Subsequently parasites were stained with primary antibody solution (polyclonal mouse anti-08.1140, 1:100 diluted in permeabilisation buffer) for 1 h, while surface controls were treated with antibody solution lacking saponin. Parasites were washed in PBS again. Anti mouse-Cy2, diluted 1:500 was added and incubated for 1 h at room temperature, followed by three washes in PBS and subsequently slides were dried. A drop of Mowiol (Calbiochem) containing DAPI (Sigma) was given onto a cover slip and was carefully placed onto the stained slide avoiding air bubbles. Slides were allowed to dry and were stored at 4 ºC in the dark. Images were acquired with the Leica SP5 confocal laser-scanning system and processed with VOLOCITY software (Improvision) at the PIF (Pathogen Imaging Facility of the University of Edinburgh).

2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism Program (Version 3.0, GraphPad Software, San Diego, California). P values below 0.05 were in general considered significant.

3. Results

3.1 *In silico* selection of novel antigen candidates from *Leishmania* spp.

A bioinformatic analysis was performed to select potential novel antigen candidates for an anti-leishmania vaccine from a proteome data set. This data set was derived from a comparative study of the proteomes of *L. mexicana* promastigotes and amastigotes (Paape *et al*., 2008). In the latter study, 509 different proteins were identified which corresponded to approximately 6 % or the gene products predicted from the *L. major* reference genome that was used for identification based on the mass spectra. In this protein set, all antigens experimentally tested in vaccines published to date were recovered. It was thus assumed that the data set was an excellent resource for the selection of novel antigen candidates for vaccines. Since experimental analysis of all 509 proteins would not have been realistic, a theoretical approach was established prioritizing requirements for antigen suitability. Protein abundance, sub cellular localization and conservation within the target species had already been tested and discussed for *Helicobacter pylori* (Sabarth *et al*., 2002), a pathogen of similar complexity. Thus, these parameters were applied in the selection process for leishmania vaccine antigens. An overview of these criteria is presented in figure 3.1 and table 3.1.

Abundance is an important parameter, since only proteins expressed above a certain threshold, are likely to be recognized by the immune system. Given the available dataset which corresponded to approximately 6 % of the leishmania proteome, the 509 proteins were considered to represent abundant polypeptides. In addition, since selective codon usage had been shown in leishmania to reflect translational bias and a codon adaptation index (CAI) had been calculated for each protein (Paape *et al*., 2008); this was used to rank the set of 509 possible candidates. All proteins have been ranked according to their CAI value with the maximum rank reaching a value of 8291.

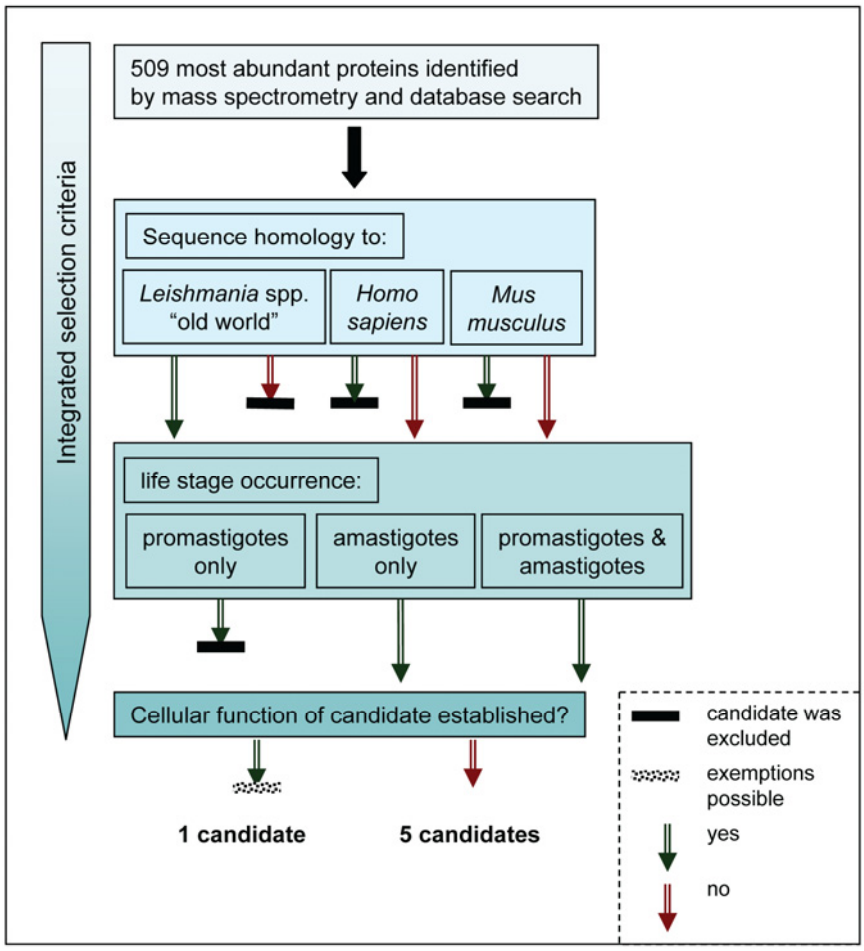

Fig. 3.1: Schematic overview of criteria applied in antigen selection process

Antigens may be more likely to be recognized as "foreign" by the immune system of vaccinated individuals if they lack homology to proteins in species targeted for vaccination (human as final target and mouse as animal model in the present case). In contrast, conservation of potential antigens within *Leishmania* species was strongly favoured. Thus, candidate proteins were further analysed bioinformatically for lack of homology to human or mouse polypeptides and for high degree of conservation between leishmania homologues (table 3.1)

A further criterion was the expression of the potential antigen in different life cycle stages. Preference was given to proteins expressed during the intracellular phase of infection (amastigotes), but, since early stages of infection after transmission of

Table 3.1: Main criteria for in silico antigen selection

selected antigens	Pro-mastigotes	Amastigotes	Abundance	CAI-Value ranking	protein function	conservation among Leishmania species[#]	homology with mammals	Source
LinJ08.1140	yes	yes	yes	7800	hypothetical protein	yes	slightly homolog with *Bos taurus*	DP
LinJ09.1180	yes	yes	yes	6141	mitochondrial RNA binding protein	yes	no significant homology	DP, Ro
LinJ23.0410	no	yes	yes	5694	hypothetical protein	yes	no significant homology	DP, Ro
LinJ25.1680	yes	yes	yes	7821	hypothetical protein	yes	no significant homology	DP, Ro
LinJ35.0240	yes	yes	yes	6888	hypothetical protein	yes	no significant homology	DP, Ro
LinJ35.2290	yes	yes[*]	yes	8194	kinetoplastid membrane protein-11	yes	no significant homology	Basu et al.

[*] low expression; [#] includes *L. braziliensis*, *L. infantum* but also *T. cruzi* and *T. brucei*; DP: Paape et al., RO: Rosenzweig et al.

Table 3.2: Putative properties of the selected antigens of technical importance

selected antigens	signal peptide	GPI-anchor	transmembrane helices	predicted localisation	molecular weight	amino acids	charge at pH 7.0	isoelectric point at pH
LinJ08.1140	no	no	no	no prediction	11.4	99	4.0	8.1
LinJ09.1180	yes, aa 1-37	no	no	no prediction	26.9	242	11	10.2
LinJ23.0410	yes, aa 1-22	no	no	no prediction	14.1	118	2.0	7.8
LinJ25.1680	no	no	no	no prediction	36.7	326	1.0	6.6
LinJ35.0240	no	no	no	no prediction	14.4	119	9.5	10.6
LinJ35.2290	no		no	surface	11.2	92	-0.5	6.3

promastigotes was also considered relevant, vaccination antigen expressed in both life cycle stages were also included. In total of six antigens were selected and, as shown in table 3.1, the majority of selected antigens were found in both stages, but LinJ23.0410 was only found in amastigotes and KMP-11, which while overly expressed in promastigotes, is only present in very little amounts in the amastigote stage (Berberich *et al*., 1998). Finally, potential antigens with unknown function (hypothetical proteins) were preferred since the main goal of this work was to search and evaluate novel vaccine candidates. An exemption was made for KMP-11, which served as a reference as it had been well characterized in previous studies (Basu *et al*., 2005).[1]

Since protein identification was based on the *L. major* genome database, conservation of the proteins within *Leishmania* spp. was an absolute necessity. Moreover, the vaccine would be primarily developed for populations in India affected by visceral leishmaniasis, a DNA preparation from a patient isolate of the etiologic agent, i.e. *L. donovani* (MHOM/INI/03BHU-55) was used for gene amplification. Due to the lack of genetic information on *L. donovani* all cloning procedures were planned using the *L. infantum* genetic database (GeneDB) as reference. Homology was analysed by comparison with *L. infantum*, *L. braziliensis* and both Trypanosoma species, *T. cruzi* and *T. brucei* using comparison tools on GeneDB (table 3.1) and ClustalW. Figure 3.2 which shows an example for such an alignment that included the sequence obtained for the respective antigen from the *L. donovani* (MHOM/INI/03BHU-55) isolate. Alignments of all other antigens were included in the supplementary section (S-1) of this thesis.

In addition to the major selection criteria described above, predicted properties of selected proteins were also taken into consideration (table 3.2). Proteins, especially when being expressed on the surface of bacteria by means of autotransporters should not be too large, so that they are easily transported through the inner and outer cell membrane. Furthermore a strong positive charge may hinder transportation, and in the presence of a putative transmembrane helix proteins may become stuck in the

[1] LinJ09.1180 which, at the time of selection, was listed as hypothetical protein - possibly mitochondrial RNA binding protein 2 (MRP2), has now been experimentally confirmed as MRP2 or gBP25 (Aphasizhev *et al*., 2003).

membrane, preventing exposure on the bacterial surface. Putative leader sequences had to be removed prior cloning, so that proteins will be targeted to the surface using the cholera toxin signal peptide from the AIDA construct as explained elsewhere. All predicted protein properties of the selected antigen candidates are summarized in table 3.2.

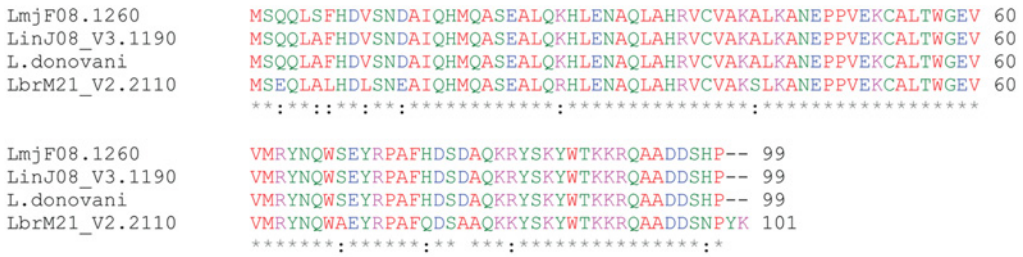

Fig. 3.2: ClustalW alignment of antigen LinJ08.1140 in different Leishmania species

Small and hydrophobic amino acids are shown in red, acidic amino acids in blue, basic amino acids in magenta and amino acids with hydroxyl or amine side chains in green.
"*" means that the residues or nucleotides in that column are identical in all sequences in the alignment; ":" means that conserved substitutions have been observed, according to the colors explained above. LmjF = *L. major*; LinJ = *L. infantum*; LbrM = *L. braziliensis*; *L. donovani* = isolate MHOM/INI/03BHU-55

Due to the dynamic process of the genome projects and their updating in databases, GeneDB changed nomenclature for the selected antigens throughout the duration of this project. Table 3.3 gives an overview with the latest update from September 2008. In this study antigens were named according to their number in GeneDB from December 2006.

Table 3.3: Nomenclature for selected antigen candidates

L. major ID	GeneDB systematic ID from December 2006*	GeneDB systematic ID from September 2008	other previous IDs
LmjF08.1260	LinJ08.1140	LinJ08_V3.1190	
LmjF09.1120	LinJ09.1180	LinJ09_V3.1180	LinJ09.0840
LmjF23.0370	LinJ23.0410	LinJ23_V3.0420	LinJ23.0380
LmjF25.1610	LinJ25.1680	LinJ25_V3.1670	LinJ25.1480
LmjF35.0140	LinJ35.0240	LinJ35_V3.0140	LinJ35.0120
LmjF35.2210	LinJ35.2290 (KMP11-2)	LinJ35_V3.2260 (KMP11-1)	LinJ35.1840

* Antigens were named according to their number in GeneDB from December 2006

3.2 Construction of optimised live vaccines

ORF for the selected antigens were cloned to be expressed on the surface or in the cytosol of attenuated *Salmonella enterica* serovar Typhimurium and thereafter to be tested in murine models for visceral leishmaniasis. Expression plasmids allowing the expression of vaccine antigens in salmonella from previous studies (Rizos *et al*., 2003; M. Sörensen, unpublished data) were modified for this purpose as presented below.

3.2.1 Surface expression plasmids

In order to express the selected leishmania antigens on the surface of salmonella plasmid pKRI143 based constructs were generated. This plasmid carries an autotransporter (AIDA) construct which was previously used for the vaccination against *H. pylori* (Rizos *et al*., 2003; J. Schroeder, unpublished data). The expression cassette consists of a cholera toxin B signal peptide, followed by *H. pylori* antigen *ure*A, and a HA-tag encoding sequence to allow detection of fusion proteins later, all linked to the 5' sequence coding for the N-terminus of AIDA. The fusion gene is under the control of the *in vivo* inducible P_{pagC} promoter. The UreA cDNA was removed from the cassette to create an empty vector ready for insertion of leishmania antigen ORFs. For this purpose a new restriction site had to be introduced which allowed the positioning of genes of interest between the signal peptide and the HA-tag sequences followed by *aida* (fig 3.3). Primers were designed with the forward primer LS_KRI XhoI (F) starting upstream of P_{pagC} and incorporating an *Xho*I site. The reverse primer LS_SpeI/BglII (R) was positioned in a way that allowed incorporation of the signal peptide in the PCR product while the 5' overhang was used to introduce an additional *Spe*I restriction site closely followed by a *Bgl*II site (fig. 3.3A). The resulting PCR product contained the P_{pagC} promoter, followed by RBS, leader sequence and the two restriction sites. Consequently the PCR product was digested with *Xho*I and *Bgl*II and ligated into an equally processed pKRI143 plasmid.

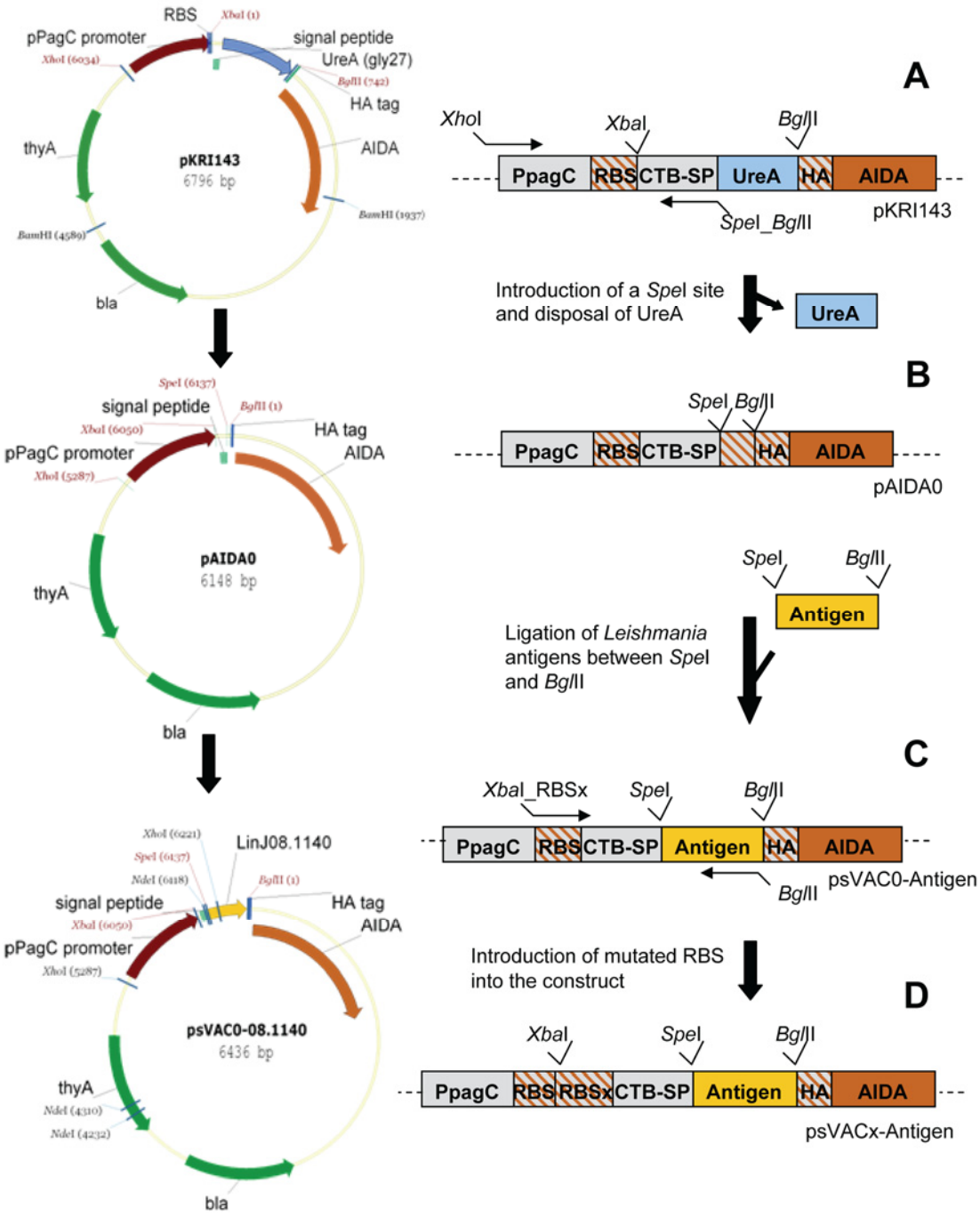

Fig. 3.3: Cloning of leishmania antigens into surface expression plasmids

Schematic overview of the cloning process that resulted in the generation of antigen free AIDA construct (pAIDA0) ready for insertion of different antigens. The subsequent cloning procedure is shown on the example of LinJ08.1140. **A**: Introduction of *Spe*I and removal of *ure*A resulting in pAIDA0 (**B**), **C**: insertion of antigen between *Spe*I/*Bgl*II and mutation of RBS resulting in **D**. **AIDA**: autotransporter involved in diffuse adherence, **bla**: beta-lactamase, **HA**: *Haemophilus influenzae* hemaagglutinin, **CTB-SP**: cholera toxin B signal peptide, **RBS**: ribosomal binding site

The resulting new plasmid contained all parts of a functional surface expression system but lacking *ureA* (fig. 3.3B). With *Spe*I an additional restriction site was introduced which allowed the ligation of leishmania antigen ORFs between *Spe*I and *Bgl*II. This new universal plasmid was named pAIDA0 (fig. 3.3).

For the ligation of antigen ORFs, gene specific primer (see table 2.1) carrying either a *Spe*I (forward) or a *Bgl*II site (reverse) at the 5' end were designed using the *L. infantum* gene sequence (GeneDB). A DNA preparation of *L. donovani* isolate MHOM/INI/03BHU-55 (Bihar, India) served as a template for PCR. The PCR products were separated on agarose gels, eluted and digested with *Spe*I and *Bgl*II, ligated into pAIDA0 plasmid (fig. 3.3C) and subsequently transformed in to *E. coli* JK321.

Importantly this strain has a deletion of the *dsb* A gene, which encodes a disulfideoxidoreductase. If present this enzyme is responsible for the formation of a stable tertiary structure of the AIDA-antigen complex, resulting in a blockage of the translocation through the outer membrane. This would lead to a rapid degradation of the fusion proteins in the periplasm. Hence lack of the disulfideoxidoreductase allows the AIDA-antigen construct to remain unfolded and be transported through the outer membrane to the bacterial surface (Jose *et al*., 1996).

Gene insertion, correct size and sequence was confirmed by colony PCR, restriction digestion and sequencing, respectively. The resulting plasmids were carrying the original RBS and were named accordingly. The nomenclature was psVAC0-antigen, with "s" standing for surface, "VAC" for vaccine, "0" referring to the wild type ribosomal binding site present and "antigen" was replaced with the relevant antigen (KMP11, 08.1140, 09.1180, 23.0410, 25.1680 or 35.0240).

3.2.2 Introduction of mutations into the ribosomal binding site

The expression of foreign antigen can highly influence the bacterial fitness of vaccine strains often leading to additional attenuation. Therefore protein expression needs to be fine tuned by introducing point mutations to the ribosomal binding site. These mutations lead to either elevated or reduced binding of the transcript to the ribosome and subsequently to a higher or lower translation efficiency of the antigen-encoding mRNA. This strategy to modulate antigen expression had been explored before and

certain point mutations had been tested (Rizos *et al*., 2003; J. Schroeder, unpublished data) and were applied in this study. Since the plasmids for cytosolic antigen expression contained already all different RBS, the antigen-encoding sequence simply needed to be replaced with one of the ORFs of the selected leishmania antigens, as described in section 3.2.1.

For surface expression strains a mutagenesis PCR was performed using the psVAC0-antigen constructs as template. The RBS was mutated with help of either of the following primer KOR_RBS4 (F), KOR_RBS6 (F) or AIDA RBS_2 (F) and gene specific primers for the reverse end. The resulting product was digested with *Bgl*II and *Xba*I and ligated into equally digested psVAC0- templates creating mutated RBS 3, 4 and 5 (fig. 3.3D). All mutations were verified by sequencing. A list of mutations and the corresponding RBS number is presented in table 3.4.

Table 3.4: Overview of all introduced point mutations into the RBS

RBS number	RBS sequence	cytosolic expression constructs	surface expression constructs	also known as, other expressions
0	AGGAG	Yes	Yes	psVAC0 pcVAC0
1	AGGAA	Yes	No	pcVAC1
2	GGGAA	Yes	No	pcVAC2
3	AGCAG	Yes	Yes	pcVAC3 psVAC3
4	AGGGA	No	Yes	psVAC4
5	AAGAA	No	Yes	psVAC5

3.2.3 Cytosolic expression plasmids

For the expression of antigens in the cytosol a series of plasmids derived from pBR322 was used (M. Sörensen, unpublished data). Plasmid pMW223 possessed the original RBS, whereas its derivates contained mutated versions in order to down regulate the expression of foreign antigens whose presence can have a negative influence on bacterial fitness.

Since these plasmids were initially designed for the expression *H. pylori* antigens, the gene for antigen HP0231 can still be found within the multiple cloning site (MCS) and needed to be removed to allow insertion of leishmania antigen encoding ORFs.

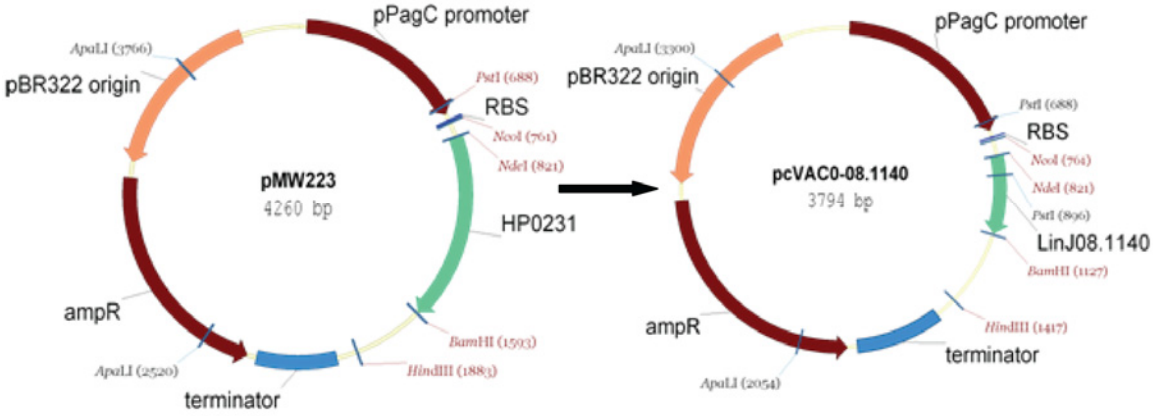

Fig.3.4: Cloning of leishmania antigen ORFs into cytosolic expression plasmids

Plasmid maps of pMW223 before digestion with *Nde*I/*Bam*HI and the resulting new plasmid pcVAC0-08.1140 after cloning the PCR fragment of the antigen (LinJ08.1140 in this case) between both restriction sites. **ampR**: ampicillin resistance cassette, **RBS**: ribosomal binding site

Figure 3.4 shows the cloning strategy on the example of ORF encoding antigen LinJ08.1140. Since none of the leishmania antigen sequences contained an *Nde*I or a *Bam*HI site, all genes of interest were cloned using these two restriction enzymes. For that purpose gene-specific primers were designed carrying an *Nde*I site (forward, also containing the ATG start codon) and a *Bam*HI site (reverse, with stop codon). A PCR was performed using *L. donovani* DNA (MHOM/INI/03BHU-55) as a template, the resulting product was purified on an agarose gel, digested with *Nde*I and *Bam*HI and ligated into pMW223 plasmid or its derivates and subsequently transformed into *E. coli* JK321.

Gene insertion, the correctness of size and sequence was confirmed by colony PCR, restriction digestion and sequencing respectively. The resulting plasmids were carrying the different mutations in the RBS and were named accordingly. The nomenclature was pcVAC0-antigen, with “c” standing for cytosol, “VAC” for vaccine, “0, 1, 2 or 3” the ribosomal binding site and “antigen” was replaced with the relevant antigen (KMP11, 08.1140, 09.1180, 23.0410, 25.1680 or 35.0240).

3.2.4 Evaluation of vaccine strains and selection of suitable candidates for protection studies in mice

To induce a protective immune response, antigens need to be administered in sufficient amounts. In the context of live vaccines, the total antigen amount delivered during vaccination is given by the product of the number of antigen molecules per bacterial cell by the number of bacteria reaching the places of antigen delivery, processing and presentation (e.g. Peyer's patches, lymph nodes and spleen). However, on the one hand high expression of foreign antigens can impair bacterial fitness and, thus, higher expression does not equal better vaccine. On the other hand, low expression levels per cell may not reduce fitness but can result in carrier antigens out-competing the vaccine antigens given the limited capacity of APC to present antigens from complex mixtures. Figure 3.5 visualizes the relation between protein expression and bacterial fitness.

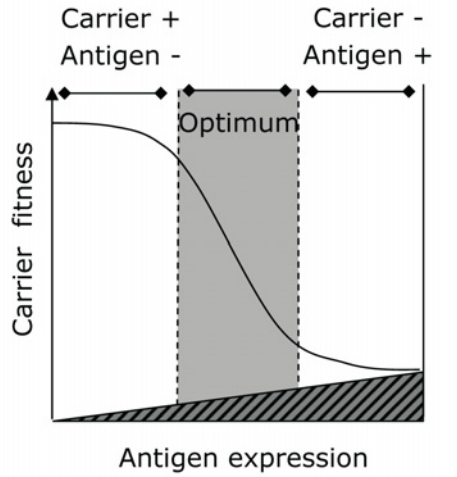

Fig. 3.5: Schematic diagram explaining the relation between antigen expression and carrier fitness

Suboptimal conditions (white area), optimal conditions (grey area), carrier or antigen can be in sufficiency (+) or insufficient (-).

For this study six antigens have been selected and salmonella vaccine strains were engineered to express the antigens either in the cytosol or on their surface. Furthermore, mutations in the RBS sequence were introduced to allow fine tuning of antigen expression levels and therefore bacterial fitness. This resulted in altogether 48 vaccine strains. A two step triage was employed, to evaluate their bacterial fitness *in vivo*, as well as antigen expression levels under *in vivo* conditions for each of the vaccine strains. This approach allowed to pre-select vaccine strains using a small number of animals before moving to vaccination experiments with larger numbers. Vaccine strains which failed to reach a certain threshold were ruled unfit and were eliminated at this stage.

The first step was to test the bacterial fitness in an *in vivo* colonisation assay. Three mice per group were oro-gastrically immunised with 10^{10} CFU of a salmonella

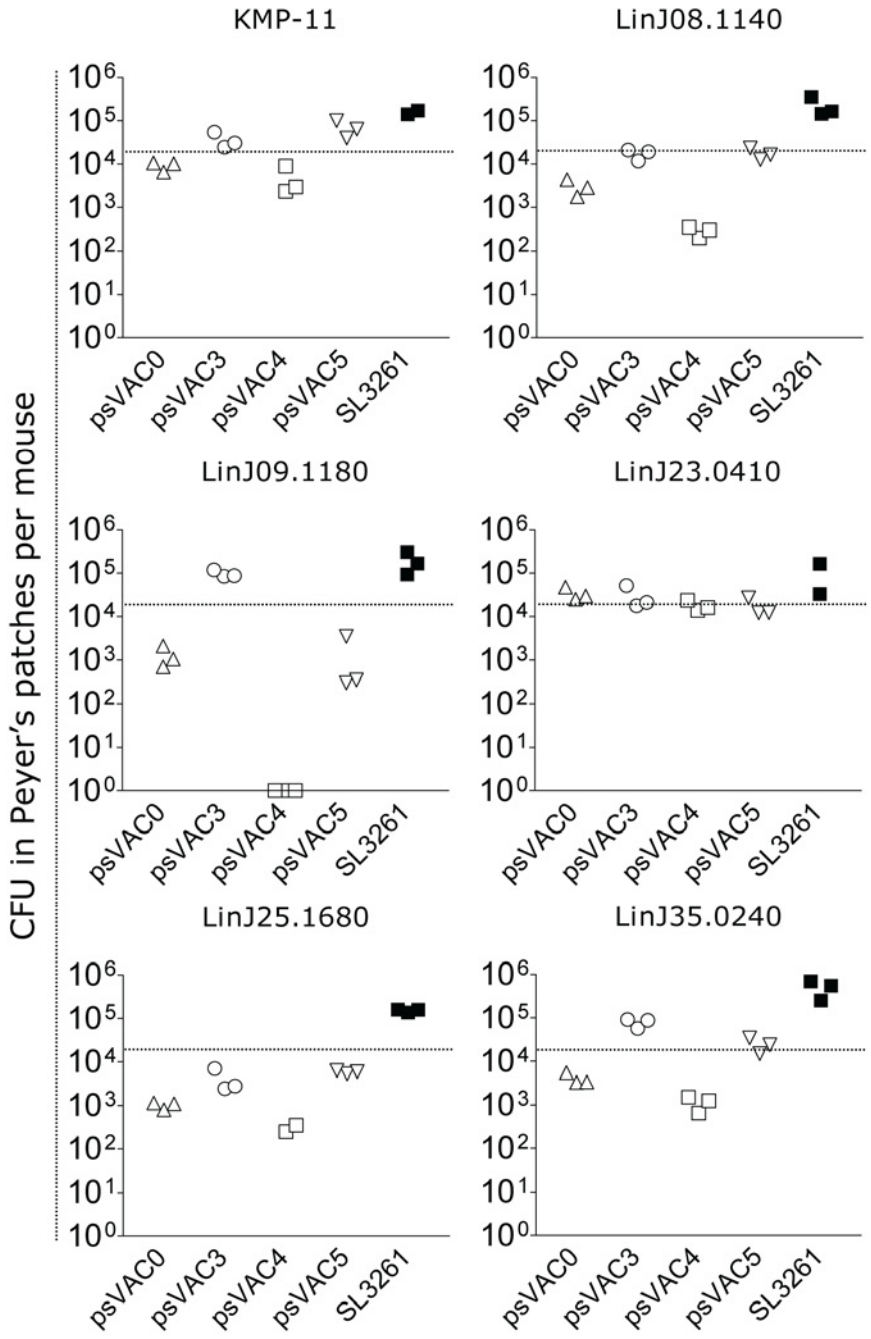

Fig. 3.6: In vivo colonisation ability of "surface" vaccine strains

Each symbol represents one mouse and symbols refer to groups vaccinated with strains carrying expression plasmids with the indicated mutations in the RBS. Control (■) was the carrier strain SL3261. Seven days after immunisation mice were sacrificed, the Peyer's patches removed, homogenized and plated on selective agar plates.

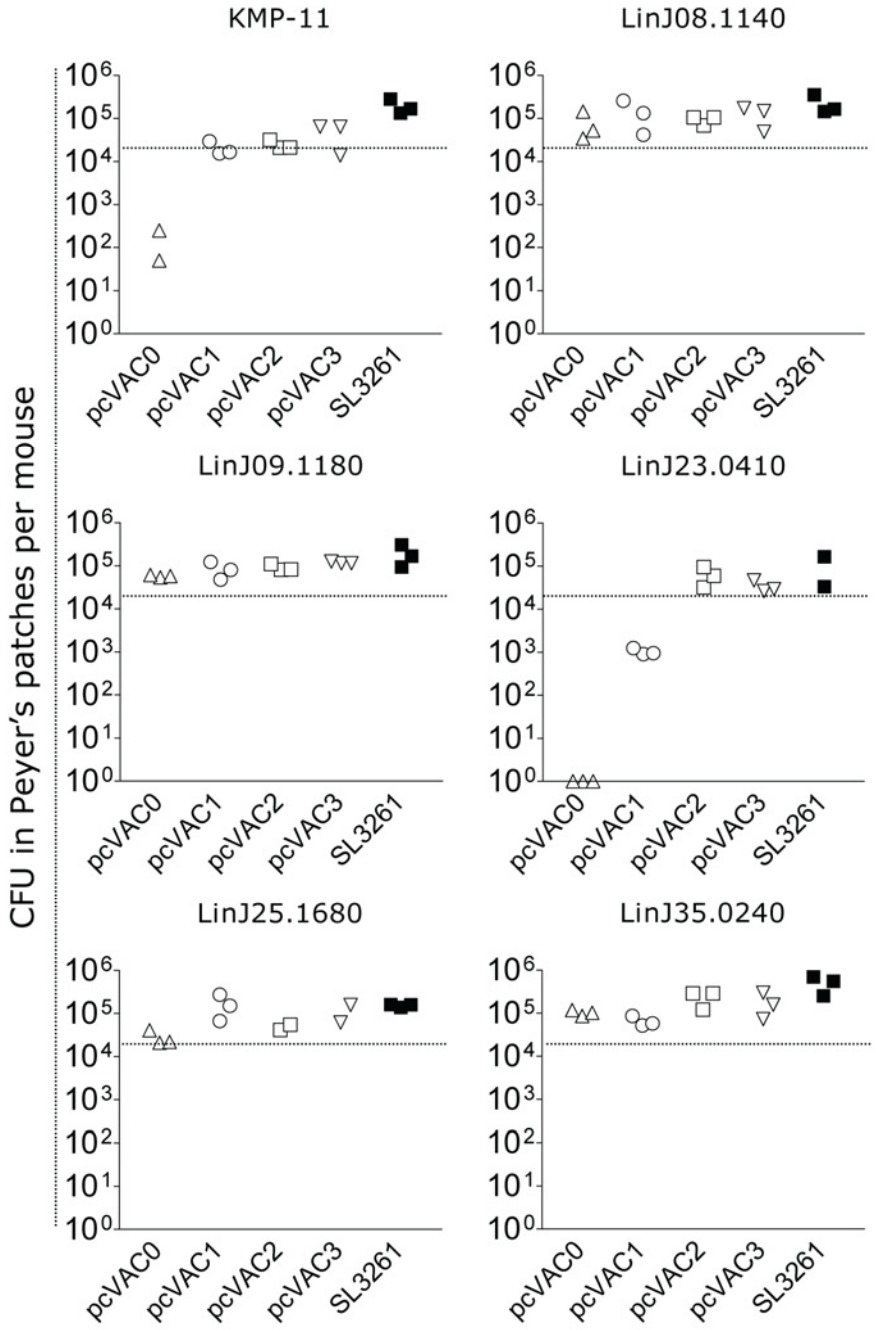

Fig. 3.7: In vivo colonisation ability of "cytosolic" vaccine strains

Each symbol represents one mouse and symbols refer to groups vaccinated with strains carrying expression plasmids with the indicated mutations in the RBS. Control (■) was the carrier strain SL3261. Seven days after immunisation mice were sacrificed, the Peyer's patches removed, homogenized and plated on selective agar plates.

vaccine strain. Seven days later mice were sacrificed and the number of colonising bacteria in the Peyer's patches was determined using selective agar plates. The results were plotted onto graphs (figures 3.6 and 3.7). It was estimated that approximately

2x 10^4 colonising bacteria were needed to present a sufficient amount of antigen to the immune system.[2] This threshold is indicated by a grey line and was ideally exceeded by selected candidates. However, expression of certain antigens (e.g. surface LinJ25.1680 or cytosolic LinJ23.0410) did overall reduce fitness with all of the strains colonizing below the threshold value or just equaling it, e.g. like surface LinJ08.1140. In these cases, strains approximating the threshold were also considered, especially when the level of antigen expression was high. The empty carrier control SL3261 showed, as expected, the highest colonisation, thus bacterial fitness. Most other strains showed a significant reduction (> 10 fold) in their colonisation abilities. However, cytosolic expression of LinJ08.1140, LinJ09.1180, LinJ25.1680 and LinJ35.0240 did not lead to a dramatic reduction in bacterial fitness (fig. 3.7). Interestingly, surface expression of LinJ23.0410, although significantly impairing bacterial fitness, showed no differences between strains harbouring mutations in the RBS (fig 3.6). Therefore antigen expression assays under conditions that mimicked the *in vivo* situation were performed in order to study the quantity of protein induced.

Antigens were expressed under the control of the P_{pagC} promoter. This promoter is regulated by the *in vivo* inducible PhoP/Q system. Upon entering the endosome of macrophages or dendritic cells, where low Mg^{2+} concentrations prevail, the PhoP/Q system is activated to initiate the expression of virulence factors and magnesium transporters, some of them under the control of P_{pagC}. Using minimal medium, supplemented to the needs of aro$^-$ salmonella (i.e. with 2,3 dihydroxybenzoic acid), and limiting Mg^{2+}, the *in vivo* situation was mimicked and protein expression was determined 3 h, 30 min after induction. Bacteria were harvested lysed and total protein amount was adjusted and equal amounts loaded onto SDS PAGE gels for analysis ("cytosolic" strains, figure 3.8) or Western blot analysis ("surface" strains, figure 3.9).

The analysis of induced cytosolic strains revealed that no antigen expression can be seen for LinJ08.1140, LinJ09.1180, LinJ25.1680 and LinJ35.0240, which is consistent with the observation that none of these strains showed a reduction in fitness compared with the SL3261 control strain.

[2] J. Schroeder, Diploma thesis (2003) Konstruktion und in vivo Evaluation von recombinanten *Salmonella*-Impfstoffträgern zur Optimierung eines anti-*Helicobacter pylori* Lebendimpfstoffs.

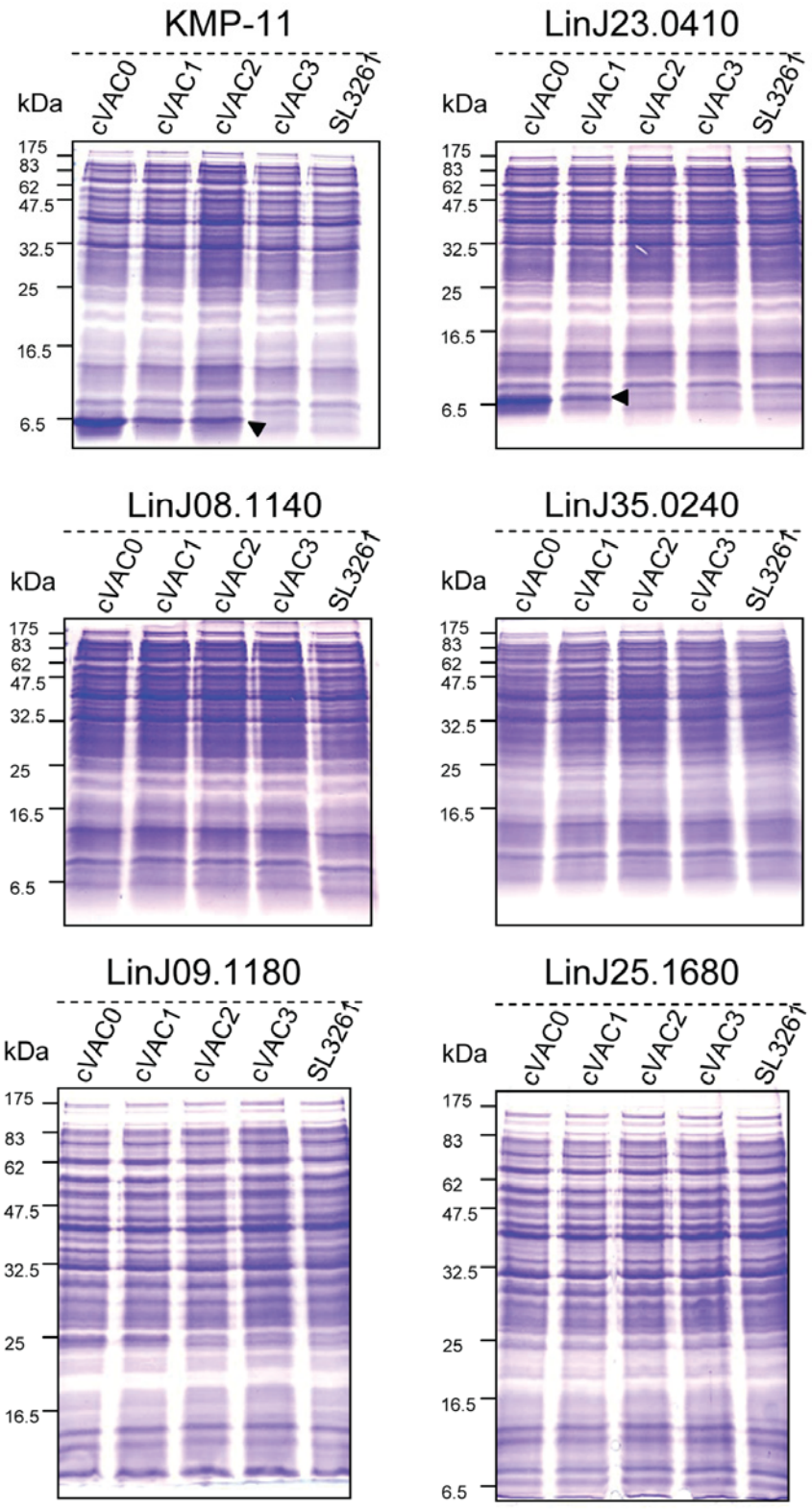

Fig. 3.8: Cytosolic protein expression after activation of P_{pagC}

Bacteria were lysed and 20 µg whole cell lysate per lane was applied, gels were stained with Coomassie. Negative control was the empty carrier control SL3261; black arrowheads indicate protein of interest.

Therefore, these strains were excluded from further analysis. KMP-11 and LinJ23.0410, however, are antigens for which cytosolic expression was seen (fig. 3.8, arrowheads), although they were running slightly below (~6.5 kDa) their predicted size (~11 kDa), which might be down to altered running performance of either marker or antigen. While the pcVAC0 version of both antigens showed the highest amount of induced antigen, the *in vivo* colonisation was rather poor. The introduction of a point mutation in the RBS of pcVAC1 or pcVAC2 resulted in less induced antigen per cell, and therewith higher bacterial fitness (fig. 3.7 and fig 3.8). Both pcVAC1 strains were selected for follow up in protection studies.

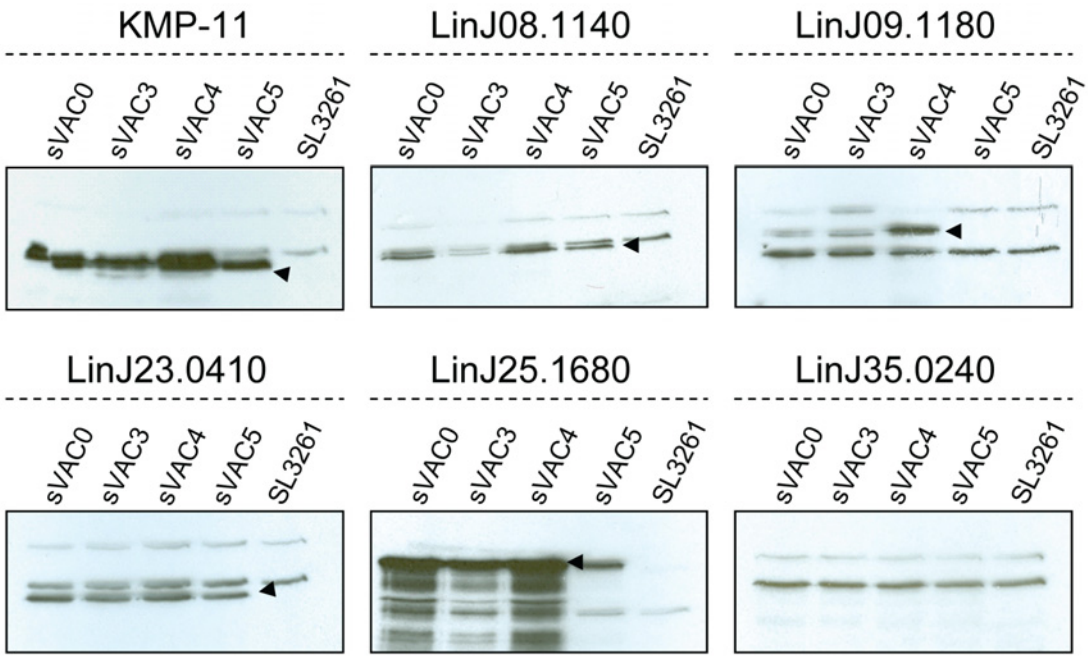

Fig. 3.9: Surface protein expression after activation of P_{pagC}

Bacteria were lysed and 20 µg whole cell lysate per lane was loaded onto SDS gels. Immunoblots were developed with an antibody against the HA-tag. Negative control was the empty carrier control SL3261; black arrowheads indicate protein of interest.

Induced expression of antigens on the surface of salmonella was visualized by detection with antibodies against an HA-tag which had been included in the construct in Western blot analyses. SL3261 itself showed two cross reactive bands at approximately 68 and 90 KDa, which were seen to be partly interfering with some of the antigen-AIDA constructs, for they have a similar molecular weight. Protein expression for LinJ35.0240 was below detection limit, although some of the strains displayed a reduced fitness. Therefore psVAC5-35.0240, which colonized on average with 2x 10^4 CFU, was selected as a representative for this antigen.

Consistent with the colonisation data, all strains expressing LinJ23.0410 showed a band with the same intensity, indicating that for unknown reasons all strains expressed similar amounts of antigen. Hence, only one strain (psVAC0-23.0410) was selected for further analysis. Induction of LinJ25.1680 on the cell surface resulted in high amounts of protein, with several degradation products. This would also explain the suboptimal colonisation ability of those strains. Two representatives (psVAC0-25.1680 and psVAC5-25.1680) have been selected to proceed into protection evaluation. Strain psVAC5-08.1140 has been selected for showing higher protein induction than psVAC3, while bacterial fitness remained level. Antigen LinJ09.1180 was highly induced in psVAC4, which unfortunately resulted in colonisation below the detection limit of 200 CFU. Therefore, psVAC3 was selected to progress into protection studies, since this strain displayed the highest bacterial fitness while expressing a detectable amount of antigen. For KMP-11 two representatives (psVAC0-KMP and psVAC3-KMP) were selected.

Altogether 10 out of 48 strains were selected for analysis of their potential to protect susceptible BALB/c mice from visceral leishmaniasis. An overview is presented in table 3.5 below.

Table 3.5: Salmonella vaccine strains selected for *in vivo* protection analysis

Antigen	Surface	Cytosol
KMP-11	2	1
LinJ08.1140	1	0
LinJ09.1180	1	0
LinJ23.0410	1	1
LinJ25.1680	2	0
LinJ35.0240	1	0

3.2.5 Purification of recombinant antigens from *E. coli*

The antigens selected (section 3.1) were cloned and expressed in salmonella carrier strains. The ability of the candidate antigens to induce a protective immune response in animal models was determined using these strains. Due to their novelty, immunological reagents like specific antibodies or purified protein were unavailable.

Therefore it was necessary to express and purify those antigens, to enable analyses of the immune response like detection of specific antibodies from serum, re-stimulation of antigen-specific T cells isolated from vaccinated individuals or for better characterization of the antigens, generation of specific polyclonal antibodies. The following section describes the antigen purification process, i.e. the generation of expression plasmids, the induction of recombinant proteins and their purification.

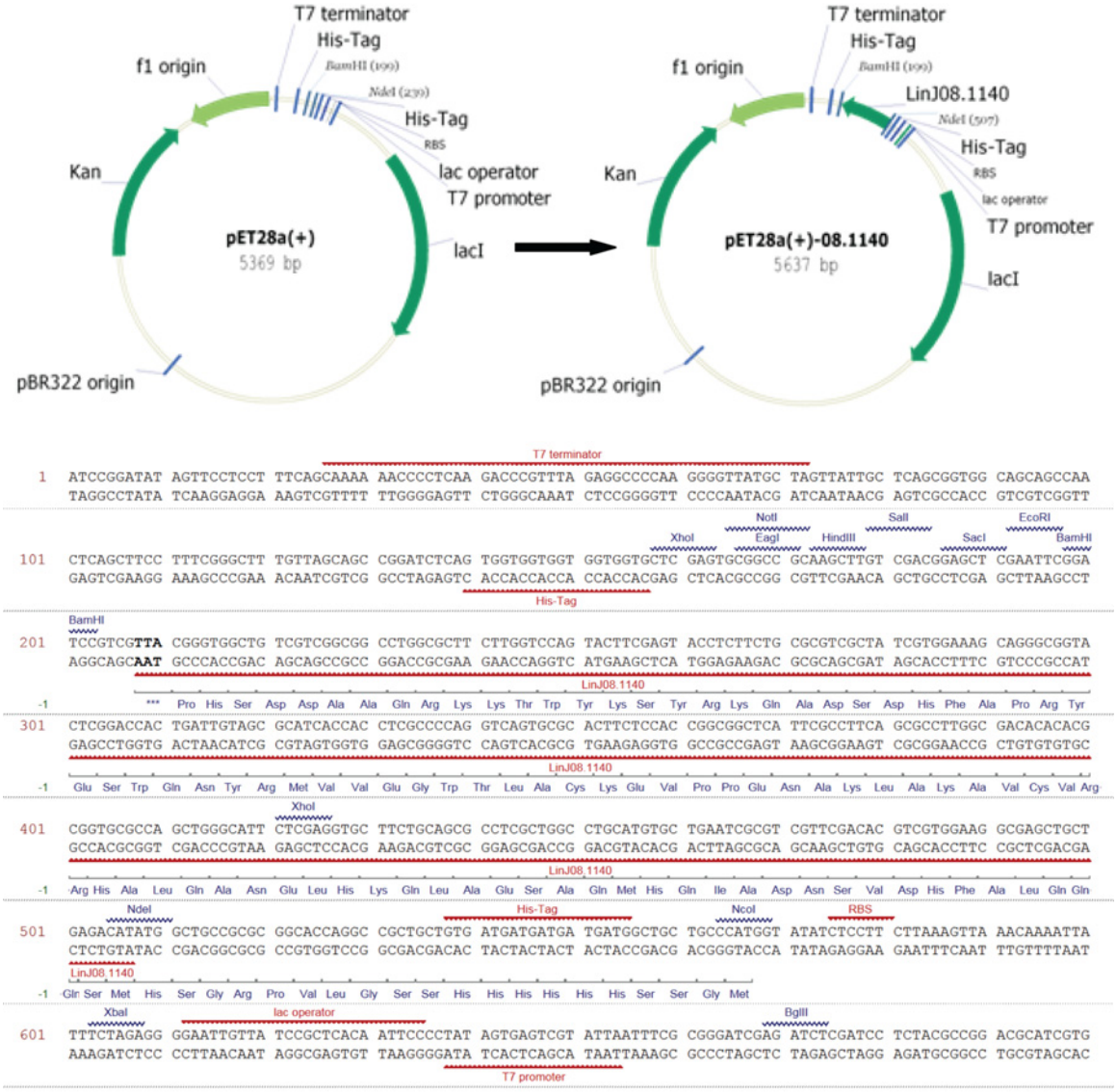

Fig. 3.10: Overview of cloning of LinJ08.1140 into pET28(+) expression plasmids

Shown are plasmid maps (**A**) before and after insertion of LinJ08.1140 gene into the expression plasmids. Below the maps the cloning site sequence is depicted (**B**), with promoter, operator, RBS, His-tags, gene and terminator sequences marked. Note: due to the stop codon in the gene of interest the second His-tag was lost.

To avoid the time-consuming process of determining purification strategies based on physical properties which may differ for each antigen, it was decided to add to antigens a hexahistidine tag which allowed purification using nickel affinity columns. For this, antigens were excised from the cytosolic expression plasmids (see section 3.2.3) using *Nde*I and *BamH*I restriction enzymes, ligated into another expression plasmid pET28(+) (Novagen) which provided an N-terminal His-tag (fig. 3.10) and subsequently transformed into *E. coli* XL-1. All constructs were confirmed by PCR, restriction digestion and sequencing.

For antigen expression, these constructs were transformed into *E. coli* strain BL21 CodonPlus (DE3)-RIPL (Stratagene), in which induction of expression by IPTG was tested on three colonies. Positive colonies showing strong protein expression were then selected to prepare bacterial stocks. All subsequent protein induction experiments were performed using these stocks. Figure 3.11 shows SDS PAGE gels of bacterial lysates prepared before and after the addition of IPTG.

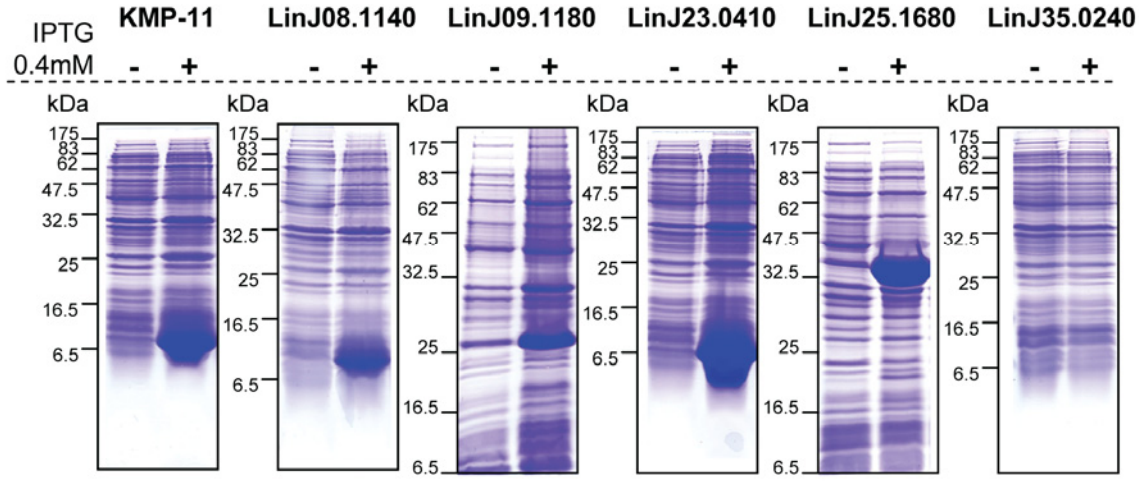

Fig. 3.11: IPTG induced expression of leishmania proteins

Recombinant protein expression was induced by addition 0.4 mM IPTG and samples were taken before (-) and after (+) induction. Samples were boiled in protein sample buffer and applied onto either 12 % SDS-gels (LinJ09.1180, LinJ25.1680) or 15 % SDS-gels (KMP-11, LinJ08.1140, LinJ23.0410, LinJ35.0240)

Titration experiments showed that most of the antigens were inducibly expressed but this required up to 1mM of IPTG. However, LinJ35.0240 antigen did not show any expression induction even at this concentration of IPTG. Furthermore, incubation at lower temperatures (22 ºC) or variation of IPTG concentration did not lead to any detectable protein expression (data not shown).

Purification of the His-tagged antigen using FPLC under native conditions was only successful for KMP-11 (see figure S-3 in Supplementary material), which was then dialysed against PBS to remove imidazole and concentrated by ultrafiltration.

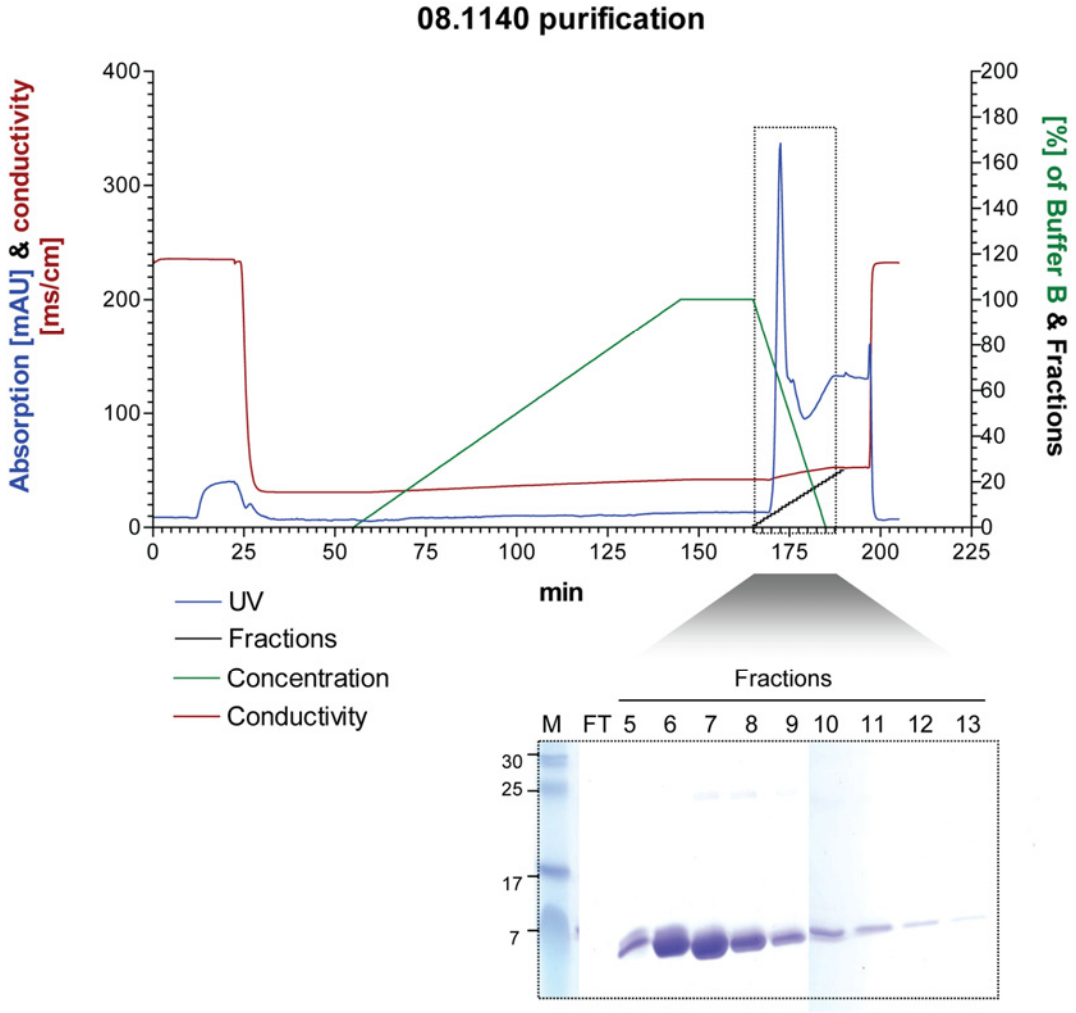

Fig. 3.12: Purification of recombinant leishmania antigens from inclusion bodies

Shown is the chromatogram of the purification of LinJ08.1140 from inclusion bodies. Fractions were collected and those representing the first peak (grey box) were applied on an SDS gel. Fractions with higher protein content were pooled and further processed. FT: flow through

The amount of protein was determined and aliquots were frozen at -80 ºC. All other antigens did not show an absorption peak during elution and consequently no positive fractions were detected by Coomassie staining of SDS PAGE gels despite being highly inducible with IPTG. It was concluded that overexpression of LinJ08.1140, LinJ09.1180, LinJ23.0410 and LinJ25.1680 resulted in the formation of inclusion bodies. After lysis, treatment with ultrasound did not dissolve the protein sample as seen with KMP-11. Instead, little round bodies were visible by light microscopy (data

not shown). Alterations in growth conditions, e.g. less IPTG, different induction times and bacterial density did not prevent the formation of inclusions (data not shown). Therefore it was decided to purify the inclusion bodies and dissolve them in urea and guanidine hydrochloride. The dissolved and denatured proteins were applied on a nickel packed affinity column and bound proteins were re-folded on-column. Eluted fractions were tested for the presence of protein by an SDS PAGE and positive fractions were pooled. An example for a successful purification of proteins from inclusion bodies is shown in figure 3.12.

Imidazole has immuno-stimulatory properties and can influence the response of T cells in re-stimulation assays. To avoid this in future experiments, dialysis against TBS was performed. Unfortunately, once imidazole was removed from the solution, most of the proteins aggregated and became insoluble. However, T cell activation is mediated via MHC-peptide/TCR complexes, hence natural conformation of the antigen is not required. Therefore, the denatured protein was stored in 50 % glycerol at -20 ºC and was used for subsequent T cell assays.

In contrast, for the detection of host antibodies against vaccination antigens, it is important to have the antigen in its natural state, since antibodies are also produced against conformational determinants. In this case, imidazole was not expected to influence antigen-antibody interaction, therefore its removal was not necessary and positive fractions from the FPLC were pooled for subsequent ELISA tests. After determination of protein concentration, ELISA plates were coated with 2.5 µg antigen per well; sealed and stored at 4 ºC until further use.

3.3 Pre-clinical evaluation of novel live vaccine candidates

The most authentic animal model of experimental VL described to date (Melby *et al*., 2001) is the syrian golden hamster infected *L. donovani,* the agent causing human VL disease. While these animals develop comparable symptoms to humans (e.g.: hepatosplenomegaly, hypoalbuminaemia, hypergammaglobulinaemia, and pancytopenia (Rodrigues, Jr. *et al*., 1998)), in the end succumbing to disease, the lack of specific reagents and no access to this model in the UK excluded its choice.
Other, more convenient models are mice, where immunological reagents are readily available. The lack of animal facilities equipped for category three pathogens at the university campus prohibited the primary use of *L. donovani*-mouse infection models. However, it is known that the normally cutaneous *L. major* causes a non healing infection in susceptible BALB/c mice characterized by progressive skin lesions and visceralization of the parasites to spleen, liver and bone marrow. Therefore, this model was chosen to test vaccine efficiency against visceralizing infection.

The 10 selected vaccine candidates were therefore tested in the latter mouse model for their ability to prevent or delay visceralisation. A pilot-experiment was carried out, to

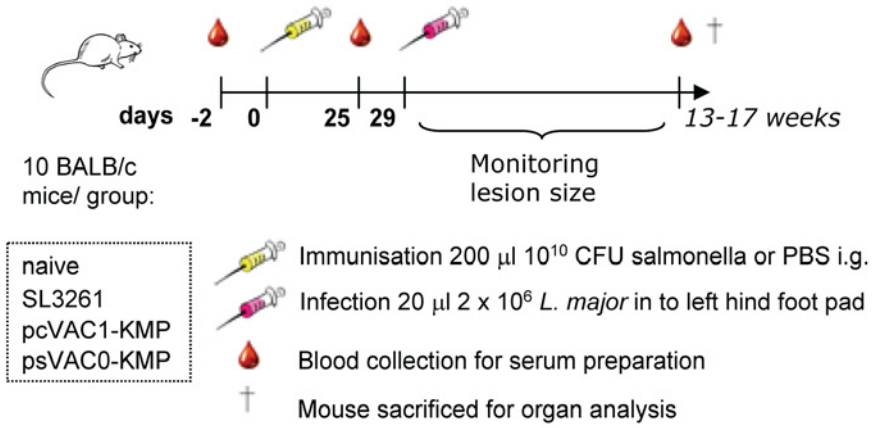

Fig. 3.13: Schematic overview of in vivo challenge
Mice have been immunised orally with 10^{10} CFU of vaccine strains or with PBS and were challenged with an s.c. injection of *L. major* promastigotes. Serum was collected before immunisation, before infection and when mice were sacrificed.

determine if salmonella were a valid carrier concept for targeting visceralisation. For this purpose salmonella strains expressing the model antigen KMP-11 on either surface or cytosol, an antigen that previously had been identified as protective (Basu

et al., 2005; Bhaumik *et al*., 2009; Delgado *et al*., 2003), were used to immunise susceptible BALB/c mice. Mice received a single dose of 10^{10} CFU of psVAC0-KMP, pcVAC1-KMP, the carrier control SL3261 or were given an equal volume of sterile PBS. Twenty nine days later all mice were challenged by s.c. injection with 2x 10^6 *L. major* promastigotes into the left hind foot pad. Figure 3.13 gives an overview of the experimental set up.

During the course of infection lesions developed in mice of all groups. Surprisingly, mice which have been given PBS showed a slow onset of disease, comparable with pcVAC1-KMP, whereas mice vaccinated with the carrier control SL3261 displayed a rapid progression in lesion development (fig. 3.14A).

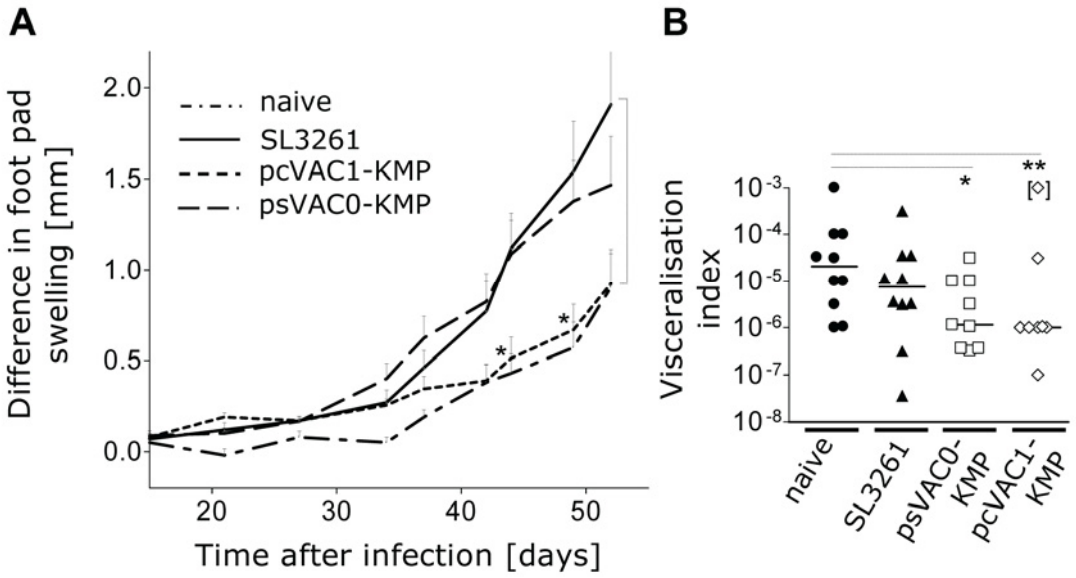

Fig. 3.14: Salmonella expressing KMP-11 reduce leishmania visceralisation in mice

A: Lesion development after infection. Error bars show standard deviation, * P≤0.05, two tailed Mann-Whitney U test.
B: Reduction of visceralisation is determined by calculating the ratio of parasites in the spleen (visceral) and the foot pad (cutaneous). Low numbers indicate reduced visceralisation. The value in brackets has been identified as outlier (Grubb's test, P≤0.05), * P≤0.05, ** P≤0.005, two tailed Mann-Whitney U test.

However, the main target parameter was reduced spreading of the parasite to visceral organs. Mice with open lesions were therefore sacrificed and parasitic burden in spleen and foot pad was determined in a limiting dilution assay, described in the methods section. Effects on visceralisation were assessed by calculating the ratio of the parasitic burden in the spleen (visceral disease) and the foot pad (cutaneous

disease) for every mouse and this visceralisation index was plotted in figure 3.14B. Interestingly, mice vaccinated with KMP-11 expressing salmonella showed significant reduction in visceralisation (pcVAC1-KMP, P=0.0097 and psVAC0-KMP, P=0.00435) compared to PBS immunised mice. The data point in brackets (pcVAC1-KMP) has been identified as an outlier by Grubb's test (P<0.05), hence excluded from further analysis. Since a small but significant vaccine effect was detected, the main study probing all ten selected strains was initiated.

To remain unbiased; the experimental design was adapted and the experimenters blinded. Vaccination groups were therefore coded. Once the first mice were sacrificed and analysed (day 48); the code was broken and the identity of the vaccine groups was revealed. An overview of this experiment is shown in figure 3.15.

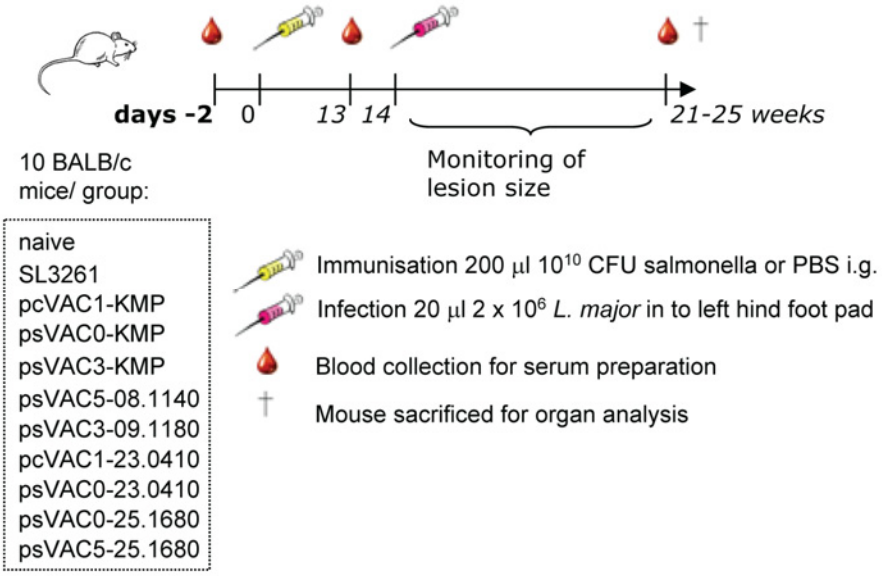

Fig. 3.15: Schematic overview of the in vivo challenge

Mice had been immunised orally with 10^{10} CFU of vaccine strains or with PBS and were challenged 14 weeks later with an s.c. injection of *L. major* promastigotes. Serum was collected before immunisation, one week before infection, at day 48 after infection and when mice were sacrificed

Female BALB/c mice were purchased from Harlan UK and immunised with the different salmonella vaccine strains (for overview see figure 3.15) or with PBS (naïve). Surprisingly, a few days after vaccination mice showed signs of murine typhoid fever (e.g. scrubby fur, weight loss and apathy), although the attenuated carrier strain SL3261 was used. Furthermore, one group, later revealed to be pcVAC1-23.0410, was exposed to additional stress caused by a defect water bottle

and as a result six out of ten mice died. The remaining animals were allowed to recover for a prolonged period of time and were subsequently infected with a dose of $2x\ 10^6$ parasites s.c. into the left hind foot pad. For the following weeks lesion development was monitored. Results are shown in figure 3.16A and in the supplementary section (S-2).

During the course of infection individuals of two groups (pcVAC1-23.0410 and psVAC5-08.1140) showed a striking reduction in lesion size compared with other vaccine and control groups. Furthermore a discrepancy between the two control groups SL3261 and naïve (shown in supplementary material S-2) was observed. Mice immunised with salmonella showed a higher foot pad swelling than naïve mice. This effect was also observed in the preliminary study. Therefore pcVAC1-23.0410 and psVAC5-08.1140 were compared to the carrier vaccine SL3261 as a reference. Both strains showed a significant protective effect ($P \leq 0.005$ and $P \leq 0.0005$ respectively) until day 48 on lesion development.

Two investigate parasitic burden in foot pad, draining lymph node and spleen, three mice per group were randomly selected (lottery) at day 48 after infection. Mice of group pcVAC1-23.0410 were not analysed, as their number was already reduced to four. The results of this analysis are shown in figure 3.16B. While no parasites were detected in the spleen of mice from group psVAC5-08.1140 and psVAC0-23.0410, and only in a limited number in mice immunised with pcVAC1-KMP, all other groups showed a higher parasitic burden and also a high variation within the group. A similar effect was observed for parasite numbers in the lymph nodes. Interestingly, mice vaccinated with psVAC5-25.1680 showed exacerbation of foot pad swelling and increased parasite numbers in spleen and lymph node. Because of the small number of animals sacrificed (n=3) no statistical analysis was performed. Nevertheless a trend that some vaccine strains conferred a protective effect was evident and therefore it was decided to investigate vaccines psVAC5-08.1140, pcVAC1-23.0410 and psVAC0-23.0410 further.

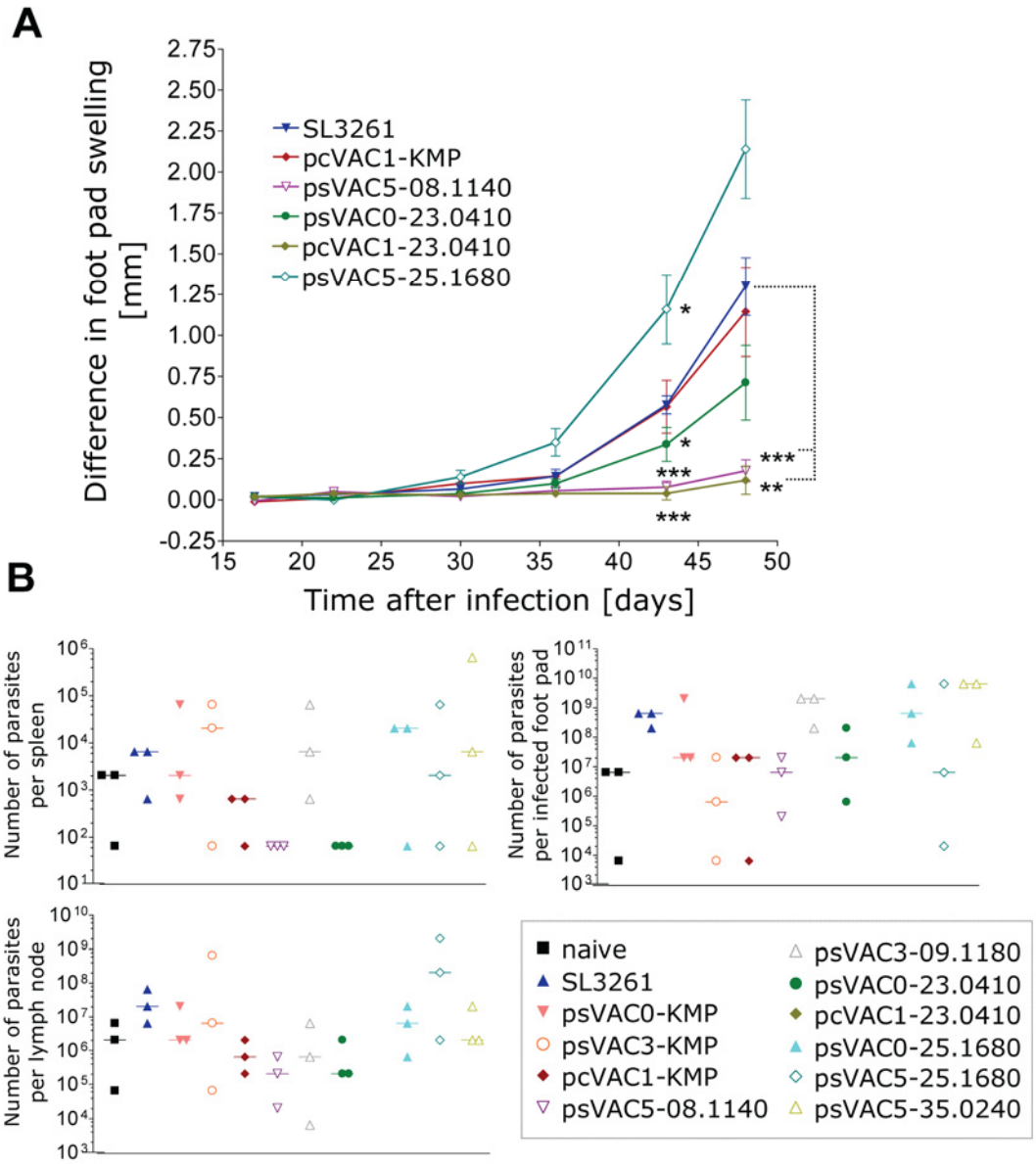

Fig. 3.16: Evaluation of candidate vaccine strains

A: Lesion development in the foot (selected strains). Error bars show standard deviation, * P≤0.05, ** P≤0.005, *** P≤0.0005 two tailed Mann-Whitney U test.
B: Parasitic burden in foot pad, lymph node and spleen. Mice (n=3) were selected at random. The detection limit was 65 for spleen and 6500 for lymph node and foot.

3.3.1 Re-evaluation of general and synergistic vaccination effect of selected vaccine strains

In the above vaccination experiments, two antigens were standing out as they significantly delayed onset of disease and were partially protecting mice from visceralisation of *L. major*. The group immunised with pcVAC1-23.0410 showed

particularly low foot pad swelling until day 48. Since this group suffered additional stress by the accidental lack of water, the vaccine effect was re-evaluated under normal conditions. Concomitantly, the positive vaccination effect of psVAC0-23.0410 and psVAC5-08.1140 were reassessed to confirm the protective nature of these vaccine strains. Both antigens reduced spreading of the parasites and since multicomponent vaccines are generally thought to be more effective, vaccine strains expressing these antigens were also combined to test for synergy.

Thus, 10 mice per group (bred at the animal facility) were immunised with either the carrier control SL3261, the vaccine strains pcVAC1-23.0410, psVAC0-23.0410, psVAC5-08.1140, or a combination of all three vaccine strains in equal amounts, in the following named vaccine allstars. A further 10 mice were treated with sterile PBS only. Blood was taken prior immunisation and upon termination of the experiment. Approximately nine weeks after immunisation mice were injected with *L. major* promastigotes. An overview of the experimental set-up is depicted in figure 3.17.

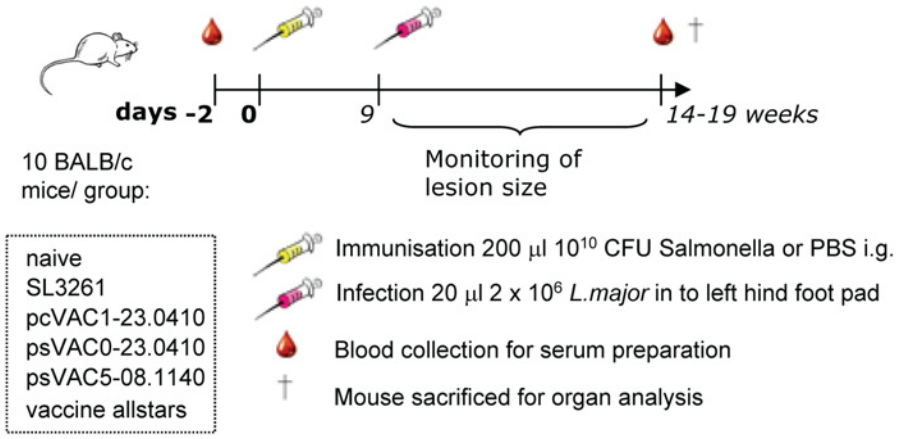

Fig. 3.17: Schematic overview of re-evaluation experiment

Mice had been immunised orally with 10^{10} CFU of vaccine strains (a combination of all vaccine strains (3x 10^{10} CFU) for vaccine allstars) or with PBS and were challenged 9 weeks later by s.c. injection of *L. major* promastigotes. Serum was collected before immunisation and when mice were sacrificed.

During the course of infection, lesion development was significantly delayed in mice which had been vaccinated either with psVAC5-08.1140 or with the vaccine allstars (fig 3.18A). Strain psVAC0-23.0410 showed a tendency to delay the onset of disease, and mice vaccinated with this vaccine showed significantly reduced foot pad swelling at day 34 ($P = 0.0097$). Immunisation with the combination of all vaccine strains reduced foot pad swelling even more ($P = 0.0003$, day 24; $P = 0.0011$, day 28; $P = 0.0006$, day 31 and $P = 0.0009$, day 34), than psVAC5-08.1140 ($P = 0.0015$, day

24; P = 0.0057, day 28; P = 0.0076, day 31 and P = 0.0041, day 34). In contrast to previous studies, mice which received PBS showed similar onset of disease as SL3261 treated animals and showed the highest parasite burden in the spleen.

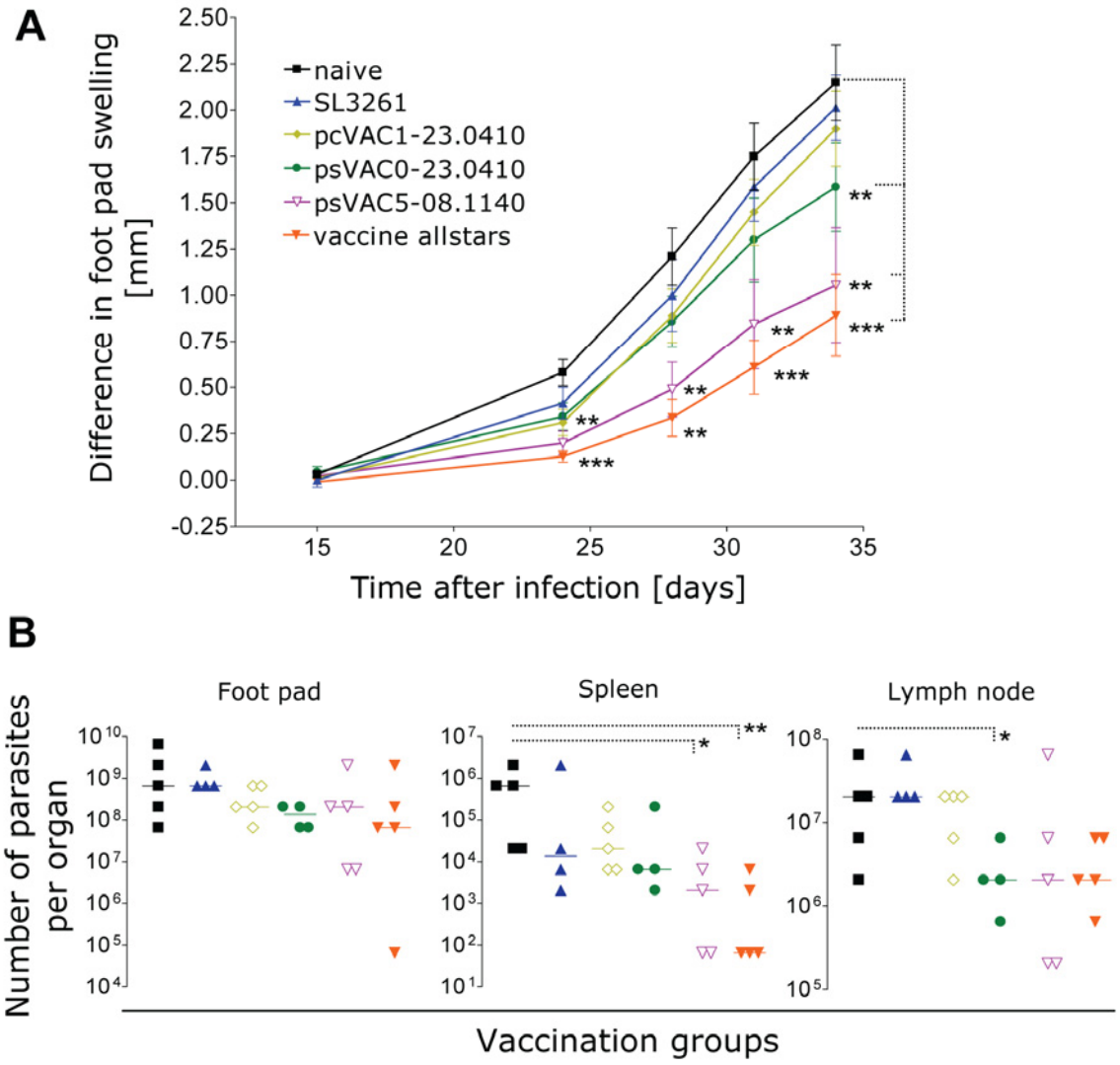

Fig. 3.18: Re-assessment of protective vaccination effects and testing for synergy

A: Lesion development in the infected foot. Error bars show standard deviation, * P≤0.05, ** P≤0.005, *** P≤0.0005 two tailed Mann-Whitney U test.
B: Parasitic burden in foot pad, lymph node and spleen. Mice (n=5) were selected at random. The detection limit was 65 for spleen and 6500 for lymph node and foot pad. * P≤0.05, ** P≤0.005, *** P≤0.0005 two tailed Mann-Whitney U test.

Before measurements commenced, five mice per group were randomly selected (lottery) for analysis of the parasitic burden in foot pad, the draining lymph node and the spleen at day 34. None of the mice cleared parasites from foot pad completely. Nevertheless a tendency in parasite reduction was observed in all vaccinated groups,

especially for psVAC5-08.1140 and the vaccine allstars (fig. 3.18B). Similar results were observed for parasite numbers in lymph nodes, but statistical analysis revealed a significant reduction in the case of psVAC0-23.0410 vaccinated mice.
However, splenic parasite burdens were dramatically reduced (up to three log) in mice vaccinated with either psVAC5-08.1140 (P = 0.0159) or the combination of all strains (P = 0.0079); of which 50 % showed no detectable parasites. Again a synergistic effect was observed, as the mean parasitic burden in the spleen of the vaccine allstars were more reduced than in any of the single vaccine groups (> 3.3 times for psVAC5-08.1140, > 31.5 for psVAC0-23.0410 and > 34.7 for pcVAC1-23.0410). Taken together this experiment showed that the vaccination effects of psVAC5-08.1140 and to a smaller extent psVAC0-23.0410 and pcVAC1-23.0410 were reproducible and moreover both antigens seemed to work in synergy in BALB/c mice.

3.3.2 Evaluation of vaccine efficiency in C57BL/6 mice

Protection against leishmaniasis is cell-mediated and requires the activation of T cells via MHC/peptide complexes. The last two studies showed that antigens LinJ08.1140 and to a certain extend LinJ23.0410 expressed by salmonella vaccines partially protected susceptible BALB/c mice from infection with *L. major*. This shows that there is a possibility that these antigens display epitopes which are recognized by MHC class I and/or II molecules of the haplotype *d*. A major requirement of vaccines in general, is that they protect the majority of a population, which normally displays a high diversity in MHC haplotypes. For that reason, a small study was performed using C57BL/6 mice which express MHC molecules of the haplotype *b*. Ten mice (obtained from Harlan UK) per group were orally immunised with 10^{10} CFU of SL3261, pcVAC1-KMP, the vaccine allstars from the previous study or with PBS. Mice were bled prior to immunisation, before infection and upon termination of the experiment. As seen in some of the previous experiments mice displayed signs of typhoid fever for approximately 2-3 weeks. They were put on a special diet in order to quicken the recovery process. Once the mice appeared fit enough (five weeks after immunisation), they were challenged with 2x 10^6 *L. major* as previously described.
C57BL/6 mice are naturally resistant against *L. major* and infection normally causes only transient symptoms (lesion development and visceralisation) and is self-healing.

Effective vaccines are expected to reduce these symptoms in comparison with control groups. Figure 3.19A shows the lesion development over the time. Groups showed a peak either at day 53 (both vaccine groups) or at day 57 (control groups). Again a discrepancy between the two control groups was observed. Effects on lesion development however were not statistically significant.

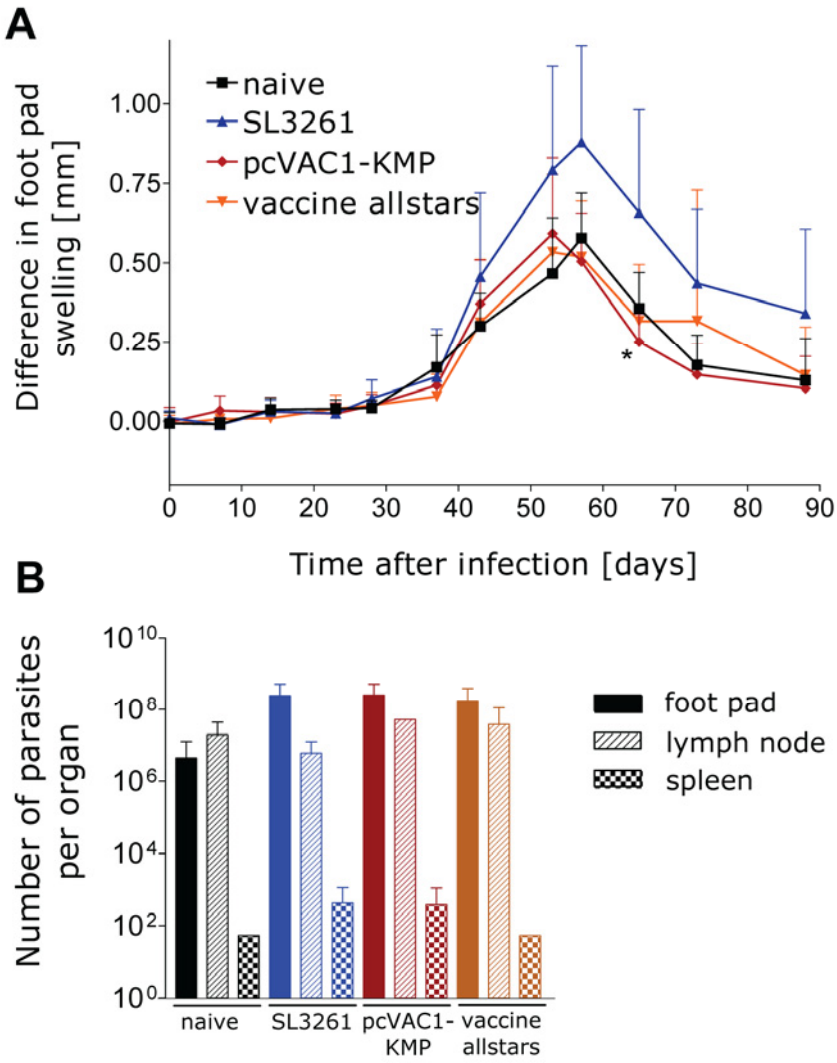

Fig. 3.19: Vaccination has only minimal effects in C57BL/6 mice

A: Lesion development in the infected foot. Error bars show standard deviation,
* P≤0.05, two tailed Mann-Whitney U test.
B: Parasitic burden in foot pad (solid), lymph node (striped) and spleen (chequered). Mice (n=5) were selected at random. The detection limit was 65 for spleen and 6500 for lymph node and foot. Error bars show standard deviation.

At day 37 after infection, five mice were randomly selected (lottery) for analysis of the parasite numbers in foot pad, draining lymph node and the spleen (fig. 3.19B). In mice vaccinated with the vaccine allstars and surprisingly in mice who received PBS

no parasites were detected in the spleen, whereas up to 1000 parasites were found in mice immunised with pcVAC1-KMP or the carrier control SL3261. No statistically significant differences were seen between the groups. Interestingly, groups that received salmonella orally showed higher parasite numbers in the foot pad (solid bar) than in the lymph node (striped bar), whereas in naïve controls it is vice versa.
Taken together, a small but not significant vaccine effect in regard to foot pad swelling and parasite burden in the spleen can be seen in mice immunised with pcVAC1-KMP or the allstars, respectively, when compared to the carrier control SL3261.

In conclusion, through *in vivo* protection studies two new antigens, LinJ08.1140 and LinJ23.0410, were identified which when expressed by salmonella carrier strains, particularly on the cell surface, partially protected susceptible BALB/c mice and also showed a tendency to reduce parasitic numbers in resistant C57BL/6 mice. To further test their suitability as vaccines against visceral leishmaniasis, another animal model utilising the agent of human VL, *L. donovani,* was investigated.

3.3.3 Evaluation of vaccine candidate strains in the *L. donovani* mouse model

Two antigens so far have been identified to partially protect BALB/c mice from infection with *L. major*. This model was chosen as a substitute for visceral leishmaniasis due to *L. donovani* infection because of the visceralising disease that *L. major* causes in these susceptible mice. However, the vaccine eventually is intended to protect humans from *L. donovani* infection. Along with other criterions antigens have been selected favouring the ones which are conserved among *Leishmania* species. Thus, the candidate vaccines were also tested in a model of *L. donovani* infection. Animal experiment project license agreements with the Home office UK, did not foresee infections with *L. donovani*. Therefore, vaccines were evaluated in collaboration with Professor Paul Kaye's research group, University of York.
Ten BALB/c mice (Charles River, UK) per group were immunised with either carrier control SL3261, pcVAC1-KMP, psVAC5-08.1140, vaccine allstars or sham-immunised with PBS as previously described. Before infection with *L. donovani* strain LV9, one mouse was sacrificed to investigate if immunisation with salmonella

resulted in splenomegally, as this would interfere with analysis at a later point. However, appearance and weight of the spleen was normal (data not shown). Mice were therefore infected with 3x 10^7 amastigotes i.v. six weeks after vaccination.

Characteristic for *L. donovani* infection in mice is the transient infection of the liver, peaking around day 28 after infection and subsequently declining. Infection of the spleen, however, leads to progressive increase in parasite numbers over the course of disease. Five mice per group were sacrificed at day 28 after infection and body, spleen and liver weights were determined in order to calculate the organ to body-mass index (BMI) for both organs (Fig. 3.20A and B). Giemsa-stained impression smears of both organs were analysed microscopically and parasitic burden was expressed in Leishman-Donovan units (LDU). Figure 3.20C and D show that vaccination significantly reduced parasitic burden in liver and spleen. Vaccination with salmonella alone reduced the mean of parasite numbers by 15 % in spleen and 44 % in liver. Cytosolic expression of KMP-11 further increased protection to 26 % (spleen) and 49.6 % (liver). Parasite numbers were even further reduced in mice immunised with salmonella expressing LinJ08.1140 (51.2 % spleen, 78 % liver) and the vaccine allstars (50.9 % spleen and 66 % liver) comprising strains expressing LinJ08.1140 as well as LinJ23.0410. Interestingly, a higher BMI for both organs was observed in mice immunised with psVAC5-08.1140 (Fig. 3.20A and B). This may have reflected inflammation rather than infection as a response of the vaccinated organism to parasitic infection. Moreover, including antigen LinJ23.0410 in the vaccine (allstars) seemed to antagonise this effect.

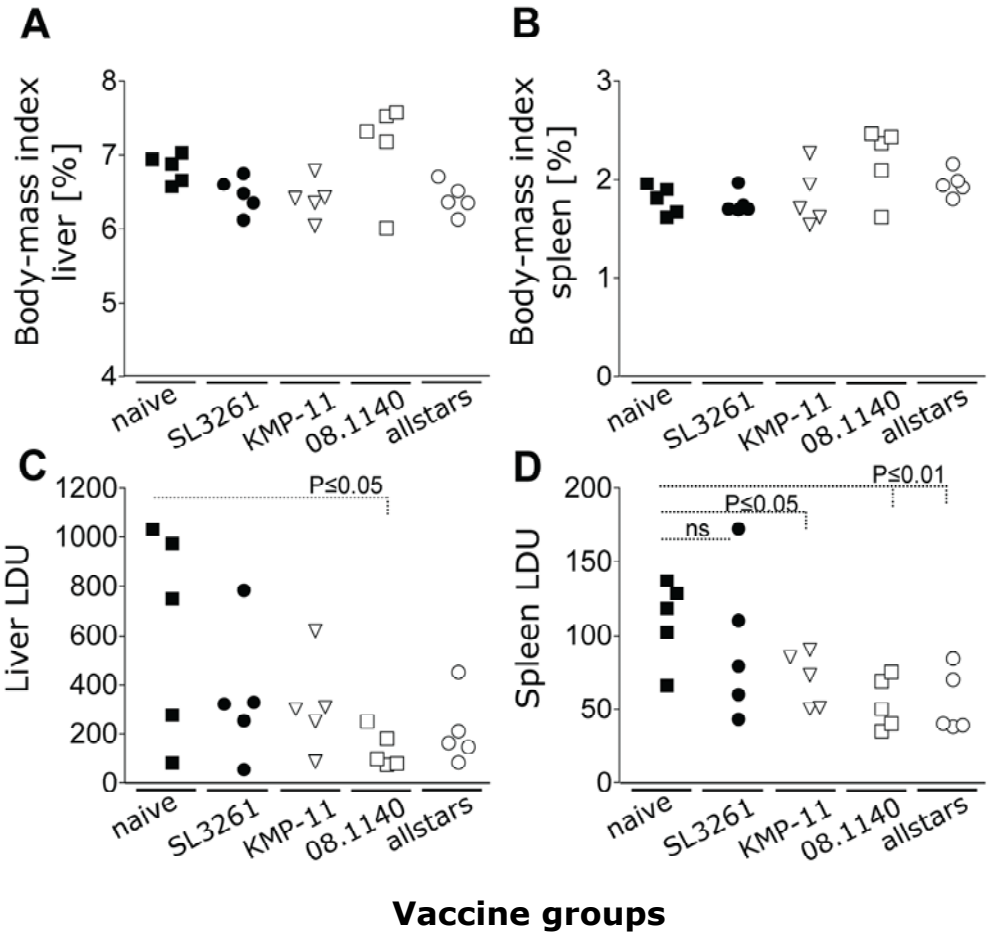

Fig. 3.20: Vaccination significantly reduced L. donovani burden in spleen and liver

Mice were killed at day 28 after infection and the body-mass index (BMI) for liver (**A**) and spleen (**B**) was calculated. Parasite count per 1000 nuclei was counted from organ impressions and related to the BMI (**C** and **D**). Statistical analysis was performed using one-tailed Mann-Whitney U test.

The remaining mice were sacrificed and analysed at a later time point (day 68) in order to determine if the vaccine is still effective at later stages of infection. Increased BMIs were observed for all vaccination groups, but in contrast to day 28 mice vaccinated with LinJ08.1140 or the vaccine allstars showed the lowest BMI (data not shown). This result is reflected in the LDU data for both organs (fig. 3.21). As expected, mean parasite numbers in the liver were declining after day 28. However, mice vaccinated with LinJ08.1140 or the allstars showed a significant reduction by 77 % and 81 %, respecitively, compared to sham-immunised mice. This effect was less pronounced in mice vaccinated with the carrier control SL3261 (48 %) or the strain expressing KMP-11 in the cytosol (36 %).

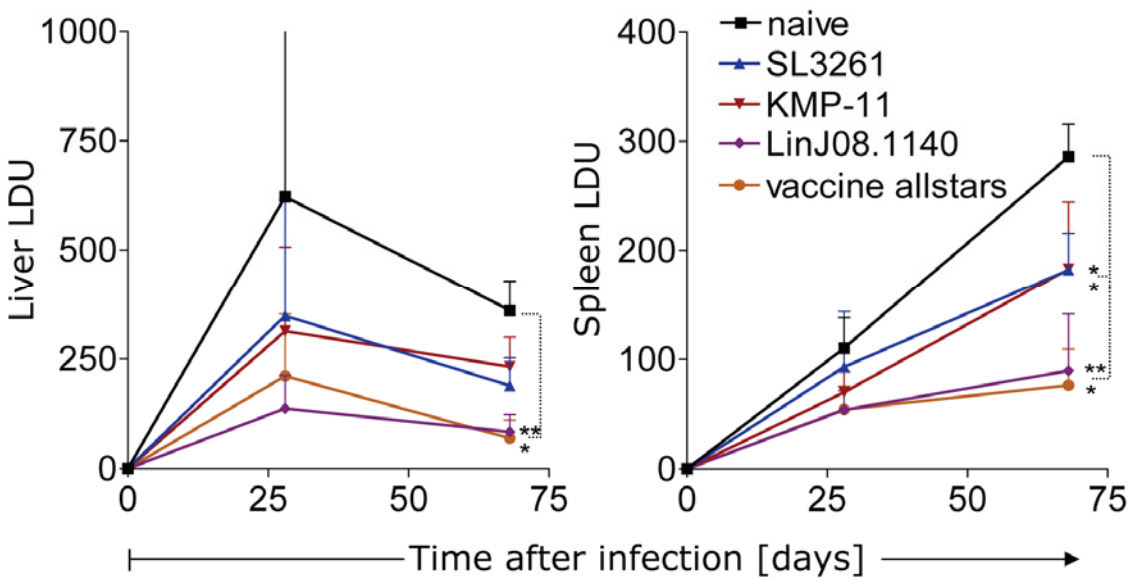

Fig. 3.21: Vaccination reduces L. donovani burden in spleen and liver over time

Mice were killed at day 68 after infection and the LDU for liver (left) and spleen (right) was calculated. Parasite numbers per 1000 nuclei was counted from organ impressions and related to the organ weight. Results from both time points are shown. Statistical analysis was performed using one-tailed Mann-Whitney U test. One mouse from group SL3261 showed at day 68 abnormally high LDU in the liver (1082) and was excluded from analysis, since it was assumed that this was likely the result of an underlying health condition (e.g. cancer) other than VL.

Parasitic burden in the spleen increased during the course of the experiment. Carrier control SL3261 and vaccine strain pcVAC1-KMP reduced the mean parasitic burden in a comparative manner (36 %) which is statistically significant (P=0.0143 and P=0.0159, respectively). However, this effect was even more distinctive in mice vaccinated with LinJ08.1140 or the vaccine allstars, where parasitic burden was reduced by 68.7 % (P=0.0079) and 73.3 % (P=0.0143), respectively in comparison to sham-immunised mice. These very encouraging results showed that prophylactic vaccination with a single dose of salmonella expressing leishmania antigens significantly reduced parasitic burden in liver and spleen compared to controls and thus vaccination effects observed against *L. major* were reproducible when using *L. donovani*, the causative agent of human VL. Measurements of appertaining immune responses were still ongoing and therefore results were not included in this work.

3.3.4 Determination of vaccine induced cellular and humoral immune responses

3.3.4.1 T cell response

Resistance against leishmaniasis infection in mice is associated with a type-1 T cell response whereas susceptibility is related to type-2 responses. Typical for Th1 responses is the production of cytokines IFNγ, TNFα and IL-2, ideally by one and the same T cell (multifunctional T cells). While IL-2 contributes to the survival of activated T cells, IFNγ and TNFα work synergistically on the activation of macrophages inducing NO synthase resulting in NO production and thus intracellular killing of parasites in infected mouse macrophages.

Ideally, a vaccine induces an antigen/parasite-specific T_H1 response which upon infection leads to rapid killing of parasites. In order to determine the response of T cells from infected or immunised and infected mice, single cell suspensions of spleens were prepared and re-stimulated for 18 hours with medium alone, or medium containing purified antigen (see section 3.2.5), or, as a control, with PMA/ionomycin for 6 h. After the addition of brefeldin A (to prevent secretion of newly synthesized proteins) for a further two hours, T cells were harvested and stained for CD3, CD4, CD8, and intracellular IFNγ, TNFα and IL-4 using specific antibodies or matching isotype control antibodies respectively. It was assumed that spleen cell suspensions contained enough APCs to present and process the antigens.
After acquisition, data was analysed using the gating strategy shown in figure 3.22. Cells were first gated for viable lymphocytes, including blasted thus slightly larger cells. These were then further analysed for the presence of CD3 molecules and positive cells were tested for CD4 and CD8 molecules respectively. $CD3^+$, $CD4^+$ and $CD3^+$, $CD8^+$ population were further gated for IFNγ and TNFα single or double producers, while no IL-4 was detected.

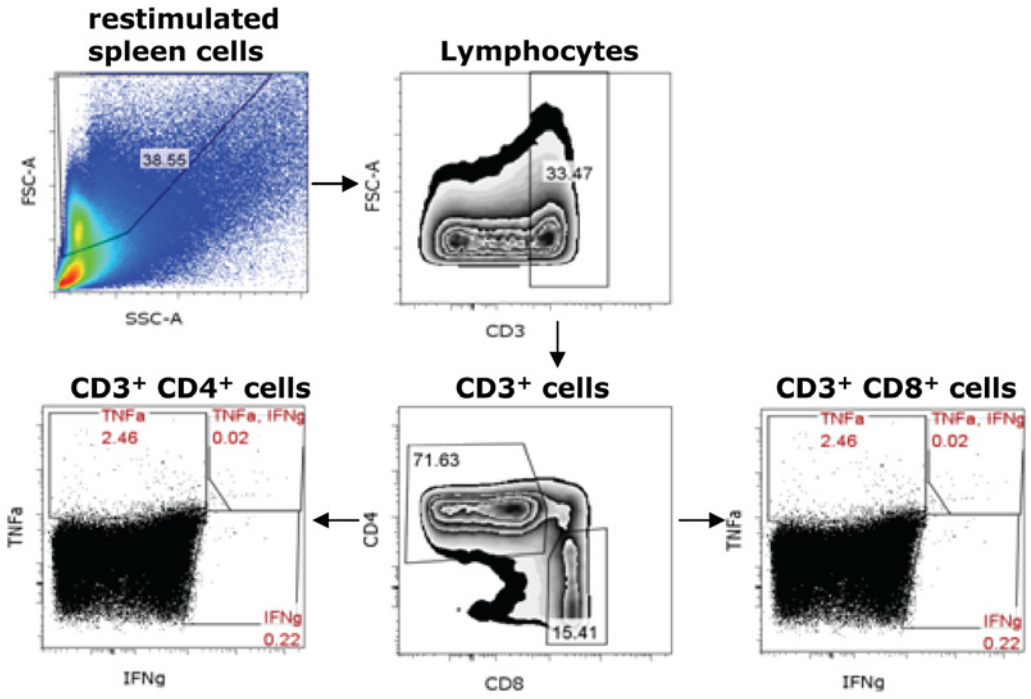

Fig. 3.22: Gating strategy for analysis of flow cytometry data

During acquisition it quickly became apparent that the sensitivity of this assay was too low. Most of the samples did not show any IFNγ/TNFα double producing T cells. It was noted, however, that mice from group psVAC5-08.1140 showed a higher frequency of $CD4^+$ and $CD8^+$ T cells (fig. 3.23). Furthermore, mice from this particular group showed

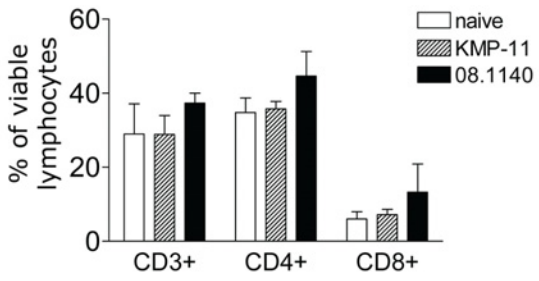

Fig. 3.23: Frequency of T cells in the spleen of vaccinated mice

Single cell suspension from spleens was restimulated with antigen for 18h and surface stained. Bars represent 3 mice per group, error bars are standard deviation of the mean.

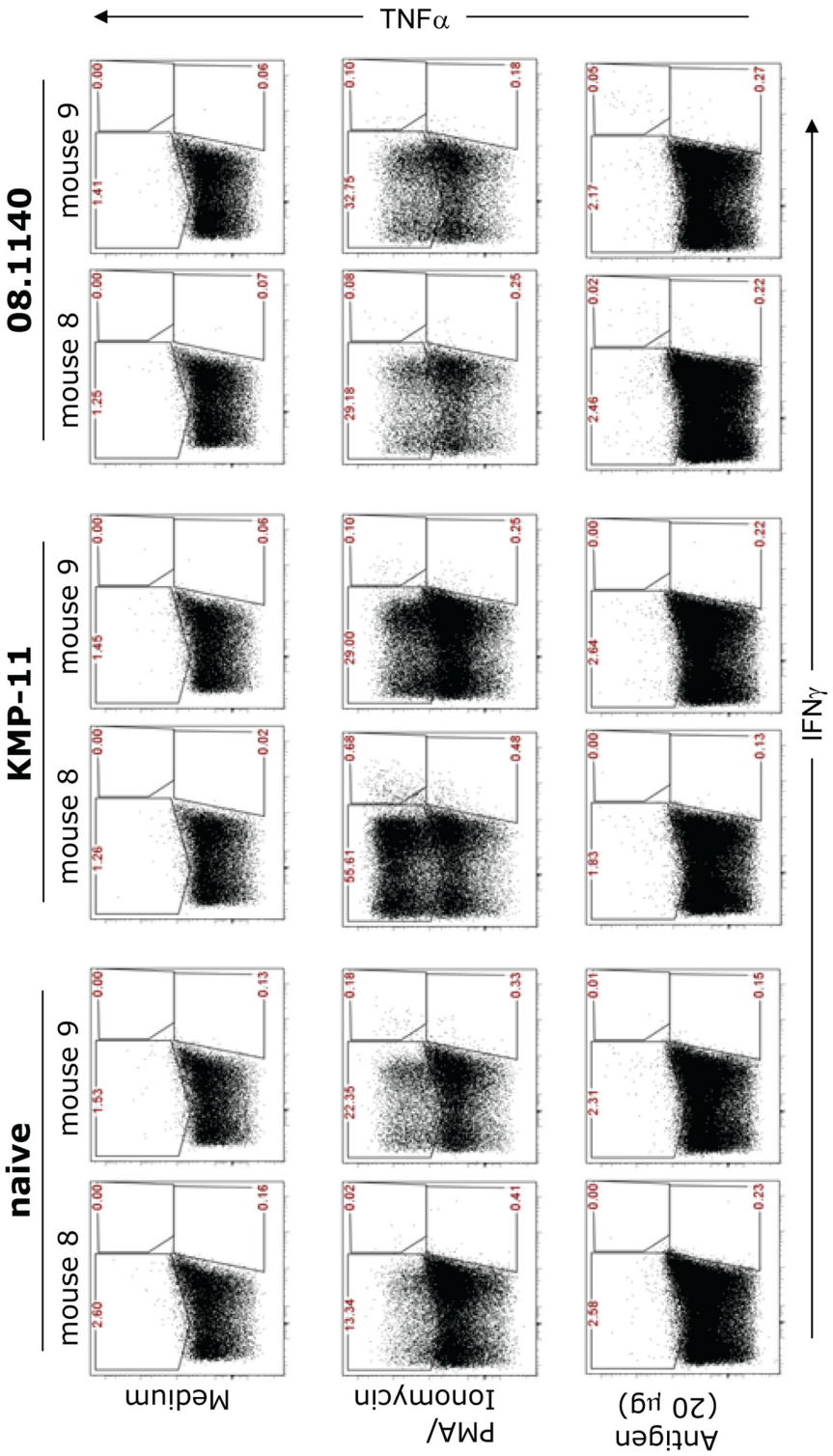

Fig. 3.24: Cytokine production of CD3$^+$, CD4$^+$ T cells after re-stimulation

a double IFNγ/TNFα-producing CD4$^+$ population (fig. 3.24), which is statistically not significant due to relatively low acquisition numbers if 2-3x 10^5. Isotype control of this group did not show any double positive T cells (data not shown).

In addition, mice from this group showed a late onset of disease, while no parasites were detected in spleen at day 48 after infection (section 3.3 and fig. 3.16). Analysis of $CD8^+$ T cells, however, did not show any significant cytokine production (data not shown).

Thus, vaccination with psVAC5-08.1140 resulted in a higher frequency of CD3, CD4 and CD8 positive T cells, of which a small fraction of $CD4^+$ cells produced IFNγ and TNFα simultaneously upon *in vitro* stimulation. This indicates that vaccination indeed resulted in the development of an antigen specific cellular response. However, firm conclusions of the T cell re-stimulation assays remained rather elusive, but may be determined with a more sensitive assay set-up.

3.3.4.2 Antibody response to vaccination

Another way to determine vaccine-induced immune responses is the measurement of antigen-specific serum IgG. During B cell development, T cell help is required and cytokines produced by the latter determine isotype switching. In general, the presence of IFNγ is correlated with IgG2a generation, whereas IL-4 produced by type-2 T cells is correlated with IgG1 isotype switch.

Mice in each *L. major* infection experiment were bled before and after challenge with *L. major* and antigen-specific antibodies in serum were determined by ELISA. When animals were bled 3-4 weeks after immunisation, immunization induced antigen-specific antibody titres were generally higher, but were already in decline at week 6 after vaccination. However, parasite infection in general boosted vaccine antigen-specific antibody titers in vaccinated mice (LinJ08.1140, cytosolic LinJ23.0410, KMP-11; fig. 3.25) and induced vaccine-antigen specific antibodies, e.g. KMP-11 (mainly IgG1), and to far lesser extent LinJ08.1140 (data not shown).

Vaccination with pcVAC1-KMP resulted in the induction of KMP11-specific antibodies, of which both isotypes IgG1 and IgG2a were detectable with similar titres. This was observed in several experiments (fig. 3.25 top panel). Infection with *L. major* did increase specific antibodies; both isotypes were again detected at similar titers in most experiments with a slight shift towards higher IgG1 titers in experiment D (fig. 3.25 top panel). Mice vaccinated with psVAC5-08.1140 on the other hand

only specific IgG2a were detected in their serum, while IgG1 remained below detection limits. This is shown

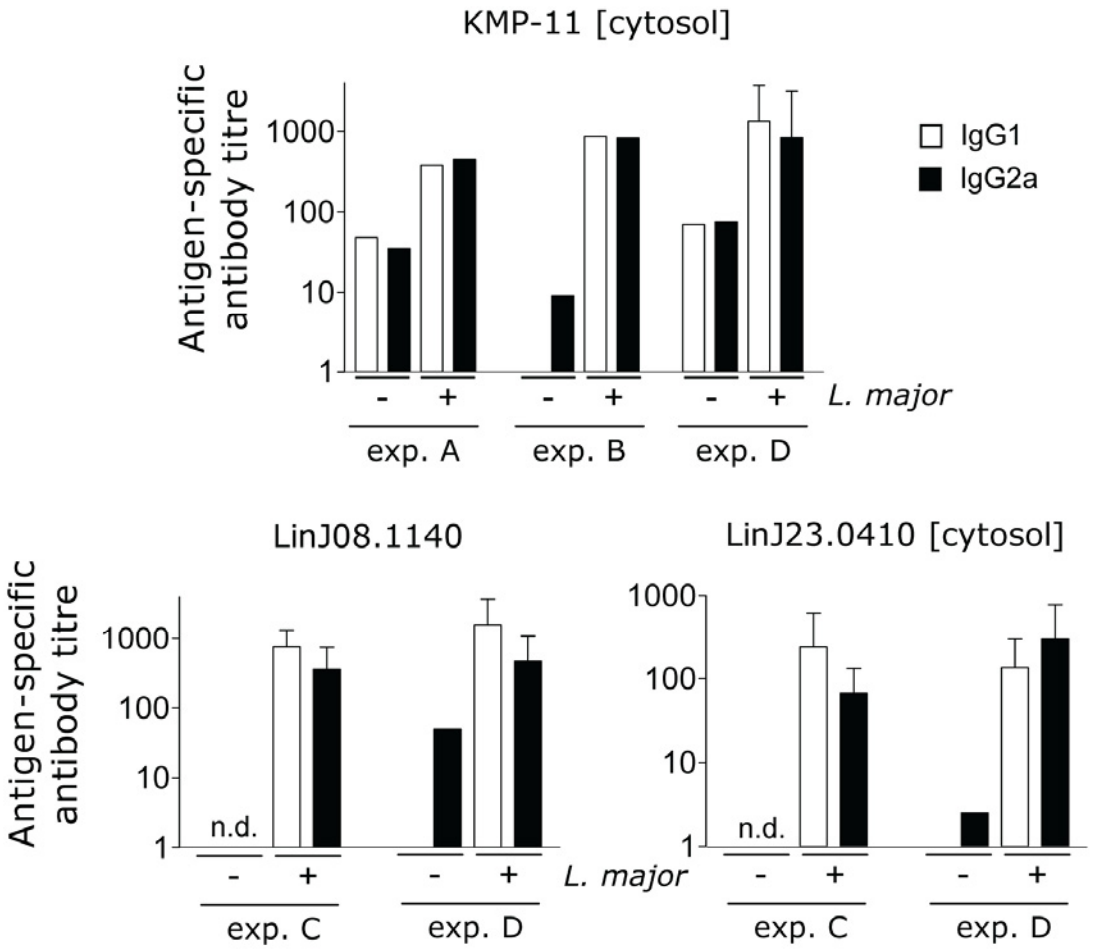

Fig. 3.25: Antigen-specific antibody response for three antigens

Serum was tested for antigen- specific antibodies of different isotypes (IgG1, white bar; IgG2a, solid bar) against vaccination antigens. Data without error bars represent serum pools of 10 mice; error bars represent standard deviation when individual analysis was carried out. n.d. not determined; exp. experiment

for experiment D (figure 3.25) and was confirmed in other experiments (data not shown). Infection with *L. major,* however, seemed to induce a shift in LinJ08.1140 vaccinated mice towards antigen-specific IgG1, through an unknown mechanism. While immunisation with psVAC5-08.1140 delayed onset of disease, the shift towards IgG1 which is connected to type-2 responses might indicate that infection-induced type-2 $CD4^+$ T cells may expand a population of B cells producing IgG1 which were below detection before challenge. Vaccination with pcVAC1-23.0410 induced low titers of specific IgG2a. While parasite infection augmented antibody

titres, the outcome was highly variable (fig. 3.25 bottom right panel). Interestingly surface expression strain psVAC0-23.0410 did not induce any specific antibody response in any of the experiments, despite showing a protective effect (section 3.3) and also failed to rise significantly after infection with *L. major* (data not shown).
In summary, immunisation of mice with salmonella vaccine strains expressing different leishmania antigens, led to an induction of specific antibodies. While the nature of the antigen seemed to determine the antigen-specific isotype induced, e.g. IgG1 versus IgG2a, parasite infection did not only augment these titres, but in some cases also led to isotype shifting towards increased IgG1 titres as seen for LinJ08.1140.

3.4 Exploiting outer membrane vesicles to augment antigen-specific immune responses

The release of outer membrane vesicles (OMV) is a common feature of Gram negative bacteria and in recent years there has been a growing interest in capitalising them for the development of affordable vaccines. OMVs have been implicated in different processes of e.g. delivery of virulence factors to target cells, intra- and inter-species communication and bio-film formation. Moreover, purified vesicles from pathogens like *Neisseria meningitis* and *Vibrio cholerae* have been tried successfully in vaccination studies. However, presently there is no evidence that other pathogens than Gram$^-$ bacteria are capable of OMV production. To take advantage of the immune stimulatory features of OMV and in order to combine them with antigens from pathogens, other than Gram$^-$ bacteria, an inducible platform was developed which should allow the expression of heterologous antigens on OMV.
Autotransporters have evolved to display on or secrete proteins through the outer membrane of Gram$^-$ bacteria. It was reasoned that proteins targeted to the outer membrane via autotransporters could also be targeted to OMVs. AIDA, the autotransporter used in this study, had already been used for surface antigen expression on salmonella vaccine strains (see section 3.2.1). AIDA fusion proteins in these strains were expressed under control of the *in vivo* inducible P_{PagC} promoter, which can also be activated *in vitro* under Mg^{2+} limitations. However, *in vitro* induction led to a decrease in bacterial growth (data not shown) and thus would

require high volume cultivation for production of OMV at a scale required for vaccine production, which disqualified this promoter for antigen expression on OMVs. Constitutive promoter activity on the other hand may damage the bacterial membranes causing bacterial death due to insertion of pore forming AIDA molecules in the outer membrane. It was therefore decided to use a recently developed propionate-inducible expression system (Lee and Keasling, 2005,Lee and Keasling, 2006), kindly provided by J.D. Keasling; to integrate the autotransporter downstream of the P_{prpB} promoter.

The AIDA cassette from pAIDA0 (see section 3.2.1) was amplified using primers designed to introduce a *Nhe*I restriction site at the 5' terminus and a *Sal*I site at the 3' terminus respectively. The resulting PCR product was digested and cloned into the MCS of an equally treated pPro18 expression plasmid, creating pMV1-AIDA (fig. 3.26). The antigens were cloned between *Spe*I and *Bgl*II as already described for surface expression constructs (see section 3.2.1). The correctness of the insert was verified using colony PCR, restriction digestion and sequencing. The resulting constructs were named according to their antigen pMV1-"antigen".

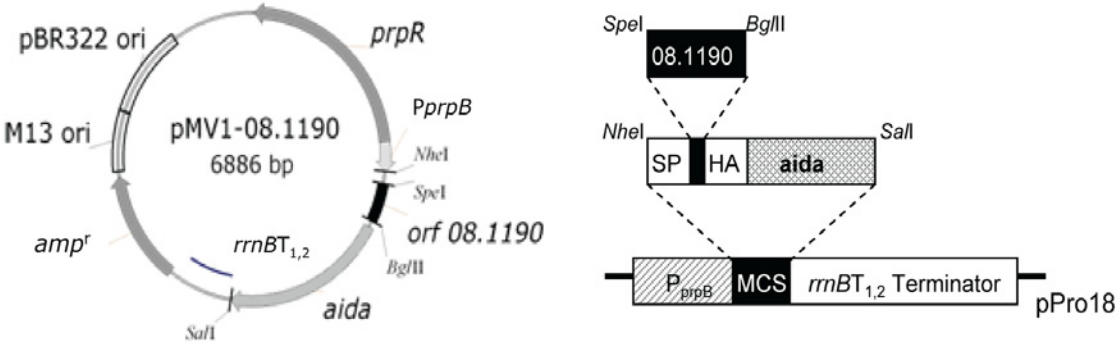

Fig. 3.26: Map of pMV1-08.1140 as a representative for all pMV1 vectors

The AIDA cassette was cloned into MCS or pBR322 derived pPro18, providing *Spe*I and *Bgl*II restriction sites, which allowed subsequent insertion of genes coding for *L. donovani* proteins. Abbreviations: ***rrnB*T$_{1,2}$** – part of strong ribosomal *rrnB* terminators, **ori** – origin of replication, **SP** – cholera toxin B signal peptide, **HA** – *Haemophilus influenzae* hemagglutinin tag, ***aida*** – autotransporter involved in diffuse adherence, ***orf*** – open reading frame (figure from (Schroeder and Aebischer 2009))

For induction experiments constructs were transformed into *E. coli* JK321. Optimisation experiments were carried out to determine the best conditions for inducing antigen expression on OMVs of such recombinant *E. coli*. For that purpose time course experiments using different concentrations of sodium propionate were

performed with pMV1-KMP and pMV1-AIDA carrying bacteria as a reference. The results indicated that a propionate concentration of 50 mM is sufficient to induce KMP-11 expression, which reached a plateau after 5 h (data not shown). In these experiments, promoter leakiness was observed for the KMP-11 construct, which is reminiscent of observations made by Lee and colleagues with the propionate inducible promoter.

All constructs were finally tested for propionate inducible antigen expression. Samples were taken prior the addition of sodium propionate and 7 h after induction. To enhance vesicle production (Kadurugamuwa and Beveridge, 1997) 50 µg/ml gentamycin was added to the culture 30 min prior harvesting. Figure 3.27 shows cell lysates of *E. coli* before and after induction. Black arrowheads indicate induced protein, while white arrowhead point out protein detected already before induction due to promoter leakiness. A clearly visible antigen expression was seen for KMP-11, LinJ08.1140, LinJ23.0410 and LinJ25.1680. However, no expression was detected for LinJ35.0240, coherent with previous expression experiments, and LinJ09.1180, which could only be detected prior induction as a result of promoter leakiness. Dot blot analysis of cell free culture supernatants (fig. 3.27, lower panel) revealed that the addition of gentamycin did indeed enhance release of expressed antigens into the supernatant and thus, likely, vesicle production. It was concluded that the propionate inducible expression system worked for the majority of antigens which can be purified for subsequent experiments.

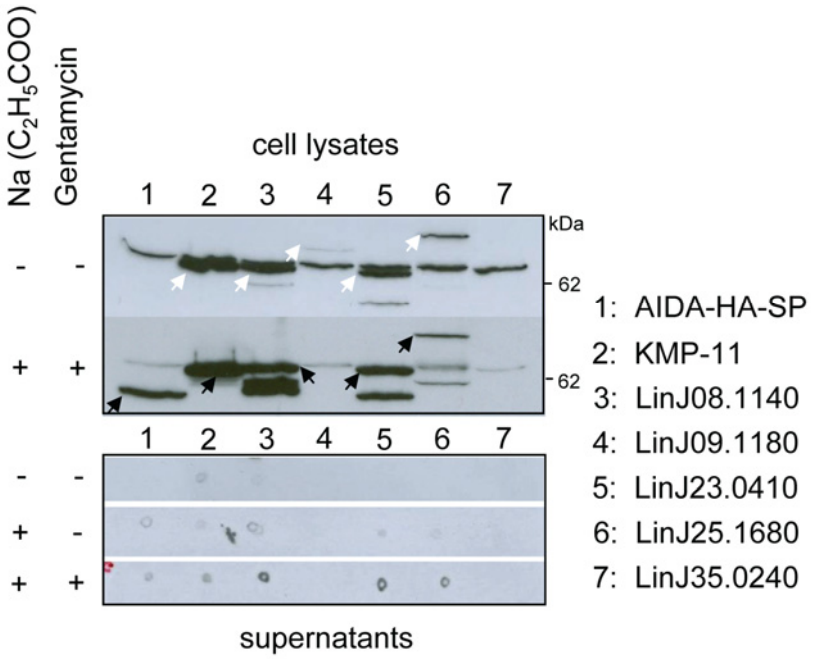

Fig. 3.27: Inducible expression of AIDA-fusion proteins for recombinant OMV production

Western blot analysis of HA-tagged fusion proteins in cell lysates (20 µg/ lane; top panels; white arrows point to protein resulting from promoter leakiness, black arrows mark induced fusion protein) and cell-free supernatants (5 µl per spot; bottom panels) prior (- -) and 8 h after addition of 50 mM sodium propionate (+ -). MV production was stimulated by gentamycin (50 µg/ml) addition for at least 30 min (+ +). Fusion proteins were detected with peroxidase-conjugated anti-HA tag antibodies.

Since vaccines containing LinJ08.1140 or LinJ23.0410 were partially protective and the KMP-11 served as a reference antigen, it was decided to test the ability of OMV expressing these antigens to boost salmonella vaccine primed immune responses. OMV were purified using the method of Moe et al., 2002 preparations typically yielding the expected 9.3 mg and 11 mg dry weight per litre of spent culture supernatant (Alaniz *et al*., 2007). Western blot analysis suggested that OMV preparations contained approximately 2.5-5 µg recombinant fusion protein per mg of dry weight (data not shown).

Twenty mice per group were immunised with SL3261, psVAC5-08.1140, pcVAC1-KMP, pcVAC1-23.0410, psVAC0-23.0410 or sham-immunised with PBS (naïve). Three weeks later mice were bled and serum was prepared and equal volumes were pooled for later determination of vaccine antigen and salmonella carrier-specific IgG2a, IgG1 and

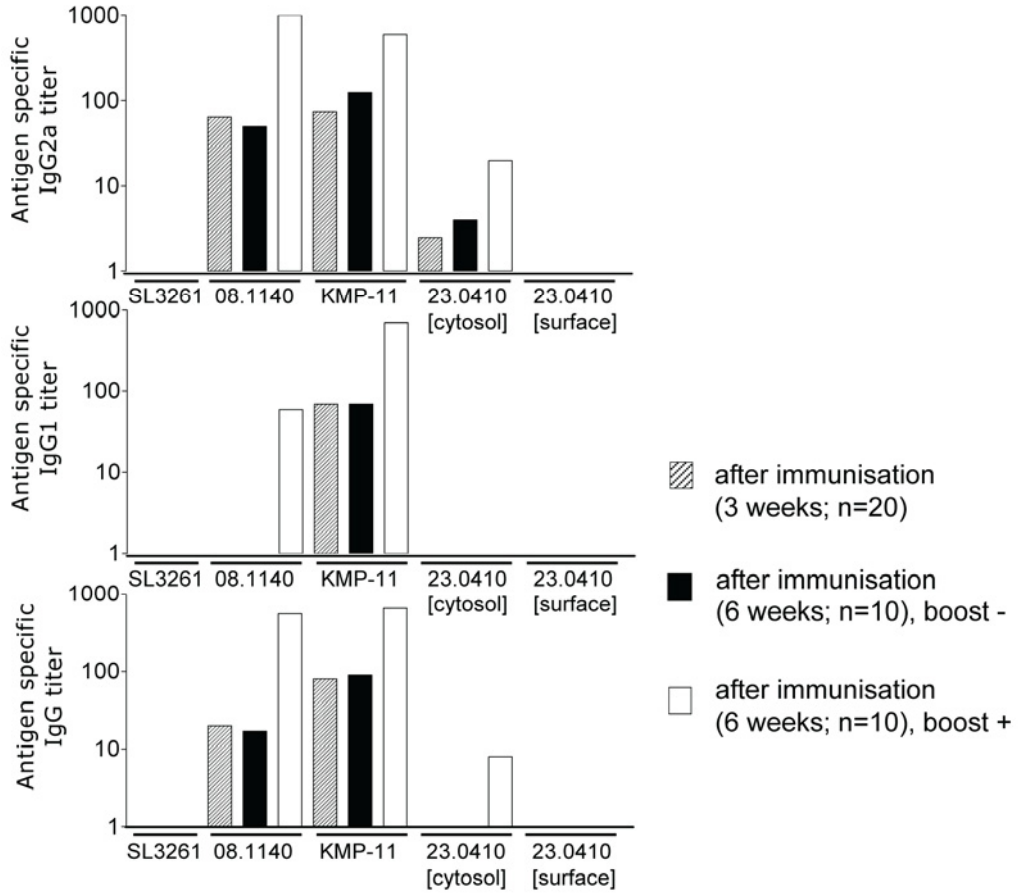

Fig. 3.28: Recombinant OMVs boost antigen specific antibodies

Specific serum antibody titres were determined in mice before (striped bar, n=20) OMV application, three weeks after a single subcutaneous booster injection of 100 µg OMV (clear bar, n=10) and 6 weeks after immunisation without boost (solid bar, n=10). Mice vaccinated with carrier control strain SL3261 were boosted with control OMV prepared from *E. coli* JK321. No cross-reacting antibodies against a mixture of KMP-11, LinJ08.1140 and LinJ23.0410 were detected. Bars represent mean antibody titres of serum pools of 20 (before OMV application) and 10 mice per group after OMV injection respectively.

Mice injected with OMV expressing KMP-11 or LinJ08.1140 showed a 6-40 fold increase of antigen specific antibodies, IgG2a as well as IgG1 in comparison to their non-boosted counterparts. Salmonella expressing LinJ23.0410 in the cytosol induced approximately 10 fold lower specific antibody titers for IgG2a compared to antigens KMP-11 and LinJ08.1140, while IgG1 remained below detection limit. Boosting resulted in an 8 fold increase of antigen specific IgG2a for this vaccine. The surface expressing strain psVAC0-23.0410 did not seem to induce any antigen specific antibodies and boosting was also not effective.

E. coli is closely related to salmonella (Falush *et al*., 2003), and boosting salmonella-immunised mice with recombinant OMV from *E. coli* may generally increase the response to the live vaccine used for priming. Focussing on expanding only the antigen-specific response of the vaccine, however, would be preferred. Therefore, the relative contribution of antigen-specific and carrier-specific antibody response to the overall vaccine induced antibody response was estimated. The respective antibody responses were plotted as proportion of their sum (fig. 3.29). The results indicated that boosting with OMV selectively expanded the response to vaccine antigens. For IgG2a, boosting with OMV carrying antigens LinJ08.1140 and KMP-11 led to an increase in the antigen specific contribution of more than 15 % to the total response to vaccination. On the other hand, priming with cytosolic LinJ23.0410 resulted in a lower specific antibody response; hence antigen-specific contribution to the overall vaccine did not exceed 1 %. Antigen-specific IgG1 for LinJ08.1140 was, although present, below the required level for titre determination (half maximal). Boosting therefore resulted in a proportional increase to 3.6 % of the total response. KMP-11 on the other hand showed a contribution of 2.5 % after vaccination which was boosted to 15 % (fig. 3.29). This indicated that boosting with a single dose of OMV can increase antigen specific immune responses.

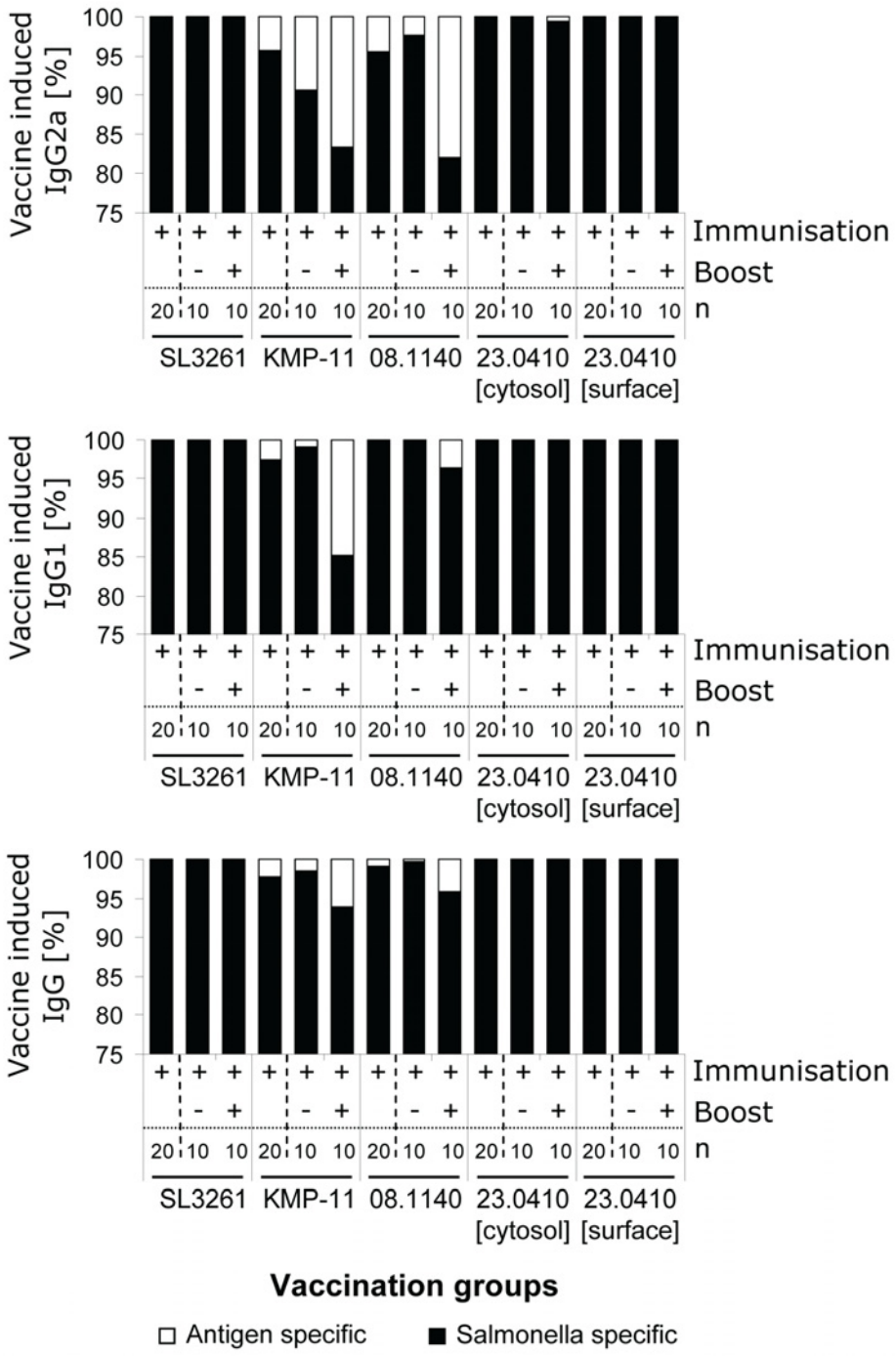

Fig. 3.29: OMV boost specifically vaccine-antigen induced antibody responses

Antigen-specific antibody titres were related to the overall immunisation-induced Ig titre (i.e. the sum of anti-vaccine antigen plus anti-SL3261 carrier response). Proportions were plotted as percentage on this overall vaccination-induced antibody titre. First bar/ group represents titres three weeks after priming (mean of n=20); second bar/group, six weeks after priming without OMV boost (mean of n=10); third bar/group six weeks after priming but three weeks after OMV injection (mean of n=10).

In order to determine if prime-boosting with recombinant salmonella and OMV vaccines increased the protective effect of the salmonella vaccines against visceral leishmaniasis, primed or primed-boosted mice were challenged with 2x 10^6 *L. major* promastigotes as described earlier. The results showed that immunisation with salmonella expressing antigens LinJ08.1140 or LinJ23.0410 reduced parasitic burden in the spleen (fig. 3.30A upper panel), although not statistically significant in this experiment. Boosting resulted in slightly increased parasite numbers in spleens of LinJ08.1140 and cytosolic LinJ23.0410 vaccinated mice (fig. 3.30A bottom), whereas a trend for reduced parasitic burden was seen for KMP-11 and surface LinJ23.0410 expressing vaccines after boosting; being statistically significant in the latter group.

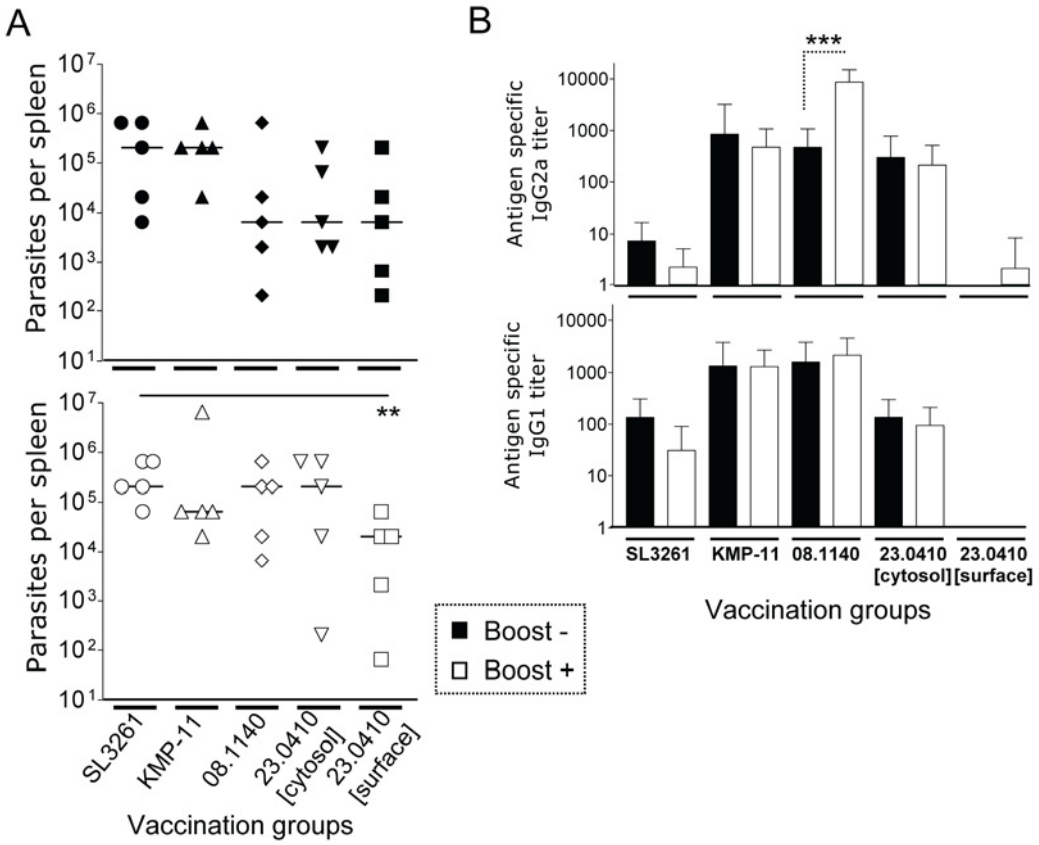

Fig. 3.30: Boosting with recombinant OMV does not increase vaccine induced protection against visceral leishmaniasis.

A: parasitic burden in the spleen of boosted (bottom, white) and non-boosted (top, solid) mice at day 35 after infection. **B**: antigen specific antibody titres from individually mice (IgG2a, top panel; IgG1, bottom panel), error bars reflect standard deviation, *** P≤0.0001, ** P≤0.001 two tailed Mann-Whitney U test.

Analysis of the antigen-specific antibody response showed an increase in both IgG isotypes for boosted and non-boosted animals (fig. 3.30B) compared to uninfected mice (fig. 3.28), indicating that all vaccination antigens are naturally immunogenic in a leishmania infection. Interestingly no difference in antigen-specific IgG1 between boosted and non-boosted groups was detected. However, mice prime-boosted with antigen LinJ08.1140 showed significantly ($P \leq 0.0001$) elevated antigen-specific IgG2a titres compared to immunised only mice.

In summary no compelling evidence was found indicating that boosting salmonella vaccine primed mice with recombinant OMV increased protection from visceral leishmaniasis. This was despite the findings that boosting with OMV increased antigen-specific antibody responses significantly.

3.5 Structural and functional analysis of LinJ08.1140

LinJ08.1140 emerged from the above experiments to be the most promising, protective antigen of those selected for this work. LinJ08.1140 represents a hypothetical protein of unknown function and no structural information is available to date. Only its sequence and predicted physical properties are known (see table 3.1 and 3.2). Therefore, in the following section first steps are presented aimed to characterize LinJ08.1140 better using bioinformatics and experimental methods.

3.5.1 Prediction of LinJ08.1140 secondary structure

The hypothetical protein LinJ08.1140 is highly conserved throughout *Leishmania* species. However, no homologues have been found in other species so far, which made the application of comparative methods for the prediction of secondary structures impossible. Therefore secondary and tertiary structures of LinJ08.1140 were predicted with *ab initio* methods. These methods are aimed at the prediction of secondary structure elements in proteins and are based on the prediction of solvent accessibility of amino acid side chains. The resulting accessibility patterns are then compared to patterns that are typically associated with certain secondary structures.

Two methods were used to analyse LinJ08.1140, the PHD method (Rost and Sander, 1993; Arnold *et al*., 2006) and PSIPRED (Jones, 1999). Both methods led to similar results. Figure 3.31 shows the secondary structure predicted by PSIPRED. LinJ08.1140 is likely to comprise three partly interrupted α-helices, linked by coil structures. 3D modelling using TasserLite (Zhou and Skolnick, 2007) resulted in a similar prediction (fig. 3.32).

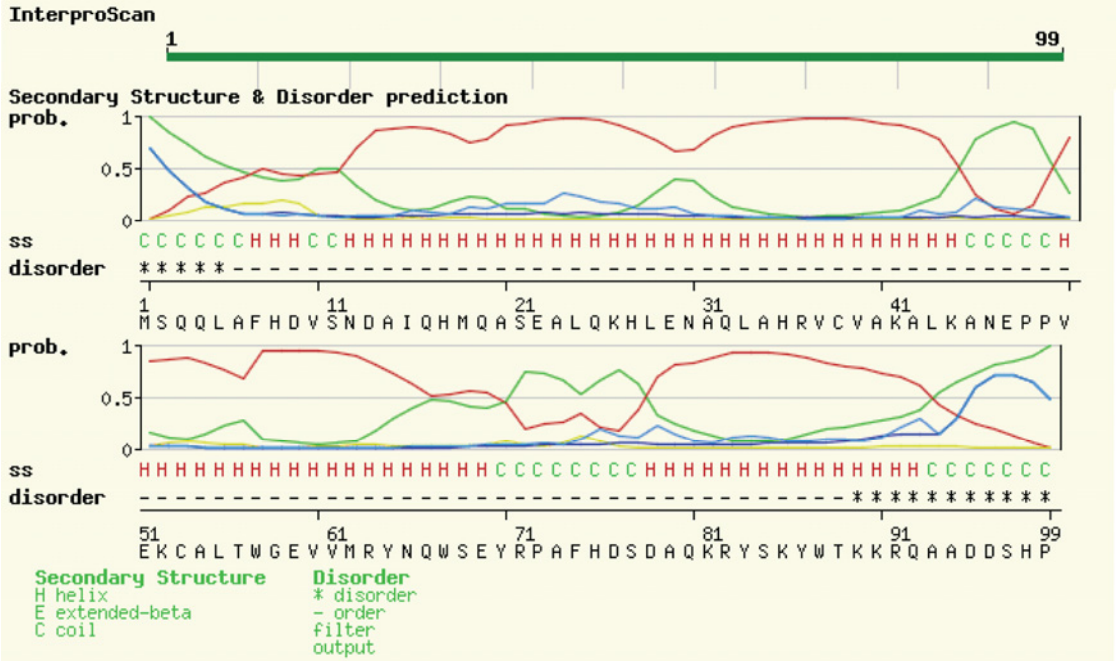

Fig. 3.31: Solving the secondary structure using ab initio prediction programs

Acronyms H, E and C are given with explanation in legend

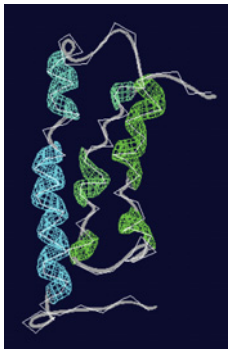

Fig. 3.32: 3D model of LinJ08.1140 using TasserLite

Alpha-helices are highlighted and colour ranges from blue (N-terminus) to green (C-terminus)

Taken together, protein structure prediction programs indicated that antigen LinJ08.1140 is a small protein comprising three α-helices and appears as a compact, globular domain.

3.5.2 Generation of a polyclonal antibody against LinJ08.1140

One mouse was immunised orally with psVAC5-08.1140 as described before. The mouse was left to recover and was several weeks later boosted with an emulsion of purified 08.1140 antigens in Freund's complete adjuvant (Sigma). Several weeks later the mouse was bled and the serum was tested for antigen-specific antibodies by ELISA using 08.1140-coated microtitre plates.

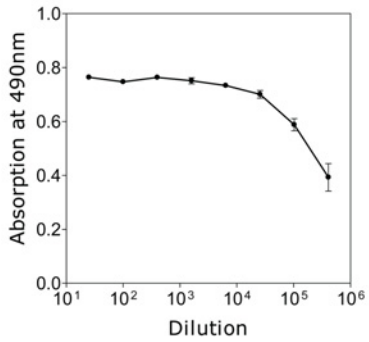

Fig. 3.33: Determination of polyclonal anti-08.1140 serum IgG

Figure 3.33 shows that very high antigen-specific antibody titres were detected in the serum of the prime-boosted mouse. A dilution of 1: 10^4 still gave maximal absorption of 0.8 at 490 nm. Subsequently the specificity of this anti-serum was tested on a range of different antigen preparations associated with this project e.g. salmonella vaccine strains expressing LinJ08.1140 or strains used for protein expression and purification.

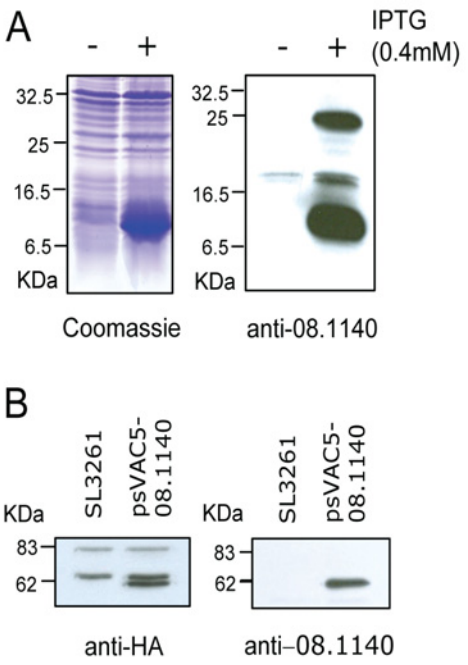

Fig. 3.34: Testing the polyclonal antibody to detect different variants of antigen LinJ08.1140

The ability to detect soluble antigen (A) and the AIDA-08.1140 fusion protein (B) was tested. **A**: *E. coli* lysates before and after induction with IPTG; left panel coomassie stained SDS gel, right panel: western blot using anti-08.1140 ($1:10^4$) and secondary peroxidase labelled anti-mouse IgG. **B**: Salmonella lysates after induction of P_{pagC}, left panel: western blot using peroxidase labelled anti-HA antibody, right panel: western blot using anti-08.1140 ($1:10^4$) and secondary peroxidase labelled anti-mouse IgG. Anti-08.1140 was additionally blocked with 100 µg SL3261-lysate.

Figure 3.34A shows a coomassie stained gel (left panel) of lysates of recombinant *E. coli* cells before and after induction of antigen-expression using IPTG (see section 3.2.5). Induced protein is clearly visible around the 11 KDa mark. Lysates were blotted onto PVDF membranes (right panel) and antigen was detected using anti-08.1140 ($1: 10^4$) and a peroxidase-labelled anti-mouse secondary antibody. To prevent unspecific binding of salmonella carrier specific antibodies, 100 µg of salmonella lysate was added to the primary antibody. Two bands became visible after detection, one at 11 KDa likely corresponding to monomeric LinJ08.1140 and another one just below 25 KDa suggesting dimer formations. Only a slight cross-reaction with *E. coli* protein around the 18 KDa mark was detected. Figure 3.34B shows Western blot analysis of cell lysates of salmonella vaccine strain expressing LinJ08.1140 on the surface and the control SL3261 after induction of vaccine-antigen expression by culture in minimal medium with low Mg^{2+} concentration (see section 3.2.5). Detection using anti-08.1140 antibody revealed a single band of the expected size for the AIDA-08.1140 fusion protein (right panel). The cross-reacting bands typically seen after detection using anti-HA antibody (left panel) were absent. However, detection of LinJ08.1140 in *L. major* lysates was not successful (data not shown). It was possible that, despite extensive washing, residual FCS used for leishmania cultivation was interfering with proper transfer and prevented transfer of leishmania proteins to the PVDF membrane.

The polyclonal anti-08.1140 antibody from mice prime-boosted with LinJ08.1140 recognised specifically the antigen from serum-free preparations such as bacterial preparations (fig. 3.34) and can therefore be used for the detection of the antigen in the parasites using fluorescence microscopy.

In order to visualise antigen LinJ08.1140 in parasites, a late log-phase promastigote culture of *L. major* 173::DsRed K2 was harvested and fixed on glass slides for subsequent staining. Parasites were permeabilised with saponin and stained either with the polyclonal antibody against LinJ08.1140 (1:50) or an equal concentration of an antiserum from a mouse immunised with SL3261 as control. To prevent cross-reaction both antibodies were blocked with salmonella lysate and detection was performed using a Cy2-labelled anti-mouse IgG antibody (1:500). As an additional control some parasites were stained with secondary antibody only. Pictures were acquired with the Leica SP5 confocal laser-scanning system and processed using VOLOCITY (Improvision) software. Figure 3.35 shows that while controls did not show any significant signal, a strong signal was observed in promastigotes stained with the anti-08.1140 antibody.

Fig. 3.35: Visualising LinJ08.1140 in L. major promastigotes using fluorescence microscopy
L. major 173::DsRed K2 promastigotes were washed, fixed, permeabelised and stained for LinJ08.1140 using the antiserum (1:50) evaluated in fig. 3.33 or an antiserum against salmonella carrier strain SL3261 of equal protein concentration. Primary antibodies were blocked with salmonella lysate and target protein was visualised with secondary Cy2 anti-mouse IgG antibody (1:500). Images were acquired with the Leica SP5 confocal laser-scanning system and processed using VOLOCITY (Improvision) software.
Arrows indicate parasites undergoing cell division.

Interestingly parasites which appear to be in the process of cell division (fig. 3.35, arrows) exhibited the strongest signal, whereas the fluorescence signal in non-dividing parasites was much weaker. In the majority of dividing cells with bright signals nuclei appeared to have already separated (not shown) while the flagellum was not fully developed and cells were still attached to each other. This indicated that the expression of antigen LinJ08.1140 by leishmania promastigotes may be cell cycle dependent and suggested a functional role in this process.

3.5.3 Mapping of MHC-I specific epitopes using *in silico* peptide prediction

Antigens are presented via molecules of the major histocompatibility complex (MHC). External antigens are processed in the phagolysosome of antigen presenting cells (APC) and loaded onto MHC class II molecules. After transport to the surface the MHC-II/peptide complex is presented to $CD4^+$ helper T cells. Antigens located in the cytosol, however, are processed in a proteasome-dependent fashion and resulting peptides subsequently are transported into the ER and loaded onto MHC class I molecules for presentation to cytotoxic T cells (CTL). The involvement of $CD8^+$ T cells in resistance against visceral leishmaniasis has been acknowledged in several studies (Basu *et al*., 2007a; Murray *et al*., 1989; Stager *et al*., 2003b). Moreover, salmonella-induced cross-presentation has been reported (Winau *et al*., 2004; Salerno-Goncalves and Sztein, 2009). Therefore, it was assumed that salmonella vaccine strains expressing leishmania antigens will, along with $CD4^+$ T cells, also activate specific $CD8^+$ T cells.

LinJ08.1140 is a newly discovered antigen, which has been shown here to be potentially protective in mouse models of leishmaniases when delivered by recombinant salmonella vaccine strains. Furthermore it would be of interest to try this antigen in other more epitope based vaccine formulations, since they allow precise initiation, regulation and control of immune responses. LinJ08.1140 comprises 99 amino acids, thus 91 putative peptides (nonamers) for MHC-I presentation. However, not all of these peptides will be immunogenic and experimental evaluation of all peptides is cost and labour intensive. Therefore, epitope prediction programs were employed to examine putative MHC-I binding peptides in LinJ08.1140 and, for

reference, KMP-11, since epitopes for the latter were predicted and tested experimentally (Basu *et al*., 2007b).

More than 30 prediction programs based on different algorithms are available on the internet. A comparative study published last year (Lin *et al*., 2008) assessed performance and reliability of MHC prediction programs using a set of model antigens. The study revealed that matrix based programs (e.g. BIMAS and SYFPEITHI) were outperformed by non-linear predictors like NetMHC which is based on artificial neural networks (ANN). Therefore analysis of LinJ08.1140 and KMP-11 was performed using NetMHC (Buus *et al*., 2003; Nielsen *et al*., 2003; http://www.cbs.dtu.dk/services/NetMHC/).

Another obstacle is the choice of appropriate MHC-I haplotypes/alleles. Genes of the MHC family are highly polymorphic with several hundred variants reported to date. Analysing all of them would exceed the scope of this work and therefore it was decided to focus on a set of haplotypes considered relevant for the major vaccine target, the population of India.

The population of India is, however, highly diverse with several thousand endogamous groups, 325 functioning languages and 25 scripts (The Indian Genome Variation Consortium, 2005). Moreover, cultural aspects like the cast system, religion and migration highly contributed to genetic diversity. However, visceral leishmaniasis is endemic in only three states in the North of India. Focussing only on Bihar, where 90 % of all cases of VL are reported, eastern Uttar Pradesh and West Bengal (Bora, 1999), the composition of ethnic populations could be better defined. Within the framework of the Indian Genome Variation Consortium it was established that the majority population of these states is Caucasian (Uttar Pradesh, Bihar and admixture in West Bengal) and Australoid (West Bengal) with mongoloid influence. This was further confirmed by another study involving Asian Indians of the Delhi area. It was suggested that the Indian population is, although essentially Caucasoid, in reality a mixture of Caucasian and oriental haplotypes/alleles (Mehra, 2000). According to this review the most common HLA class I alleles in the Indian population were A*02, A*24, A*11, A* 33 in the HLA-A locus and B*07, B*35, B*40, B*57 and B*58 in the B locus. Thus, for the purpose of predicting vaccine antigen-derived epitopes and it was decided to restrict the analysis to these most common haplotypes. Interestingly the most ubiquitous Caucasian allele

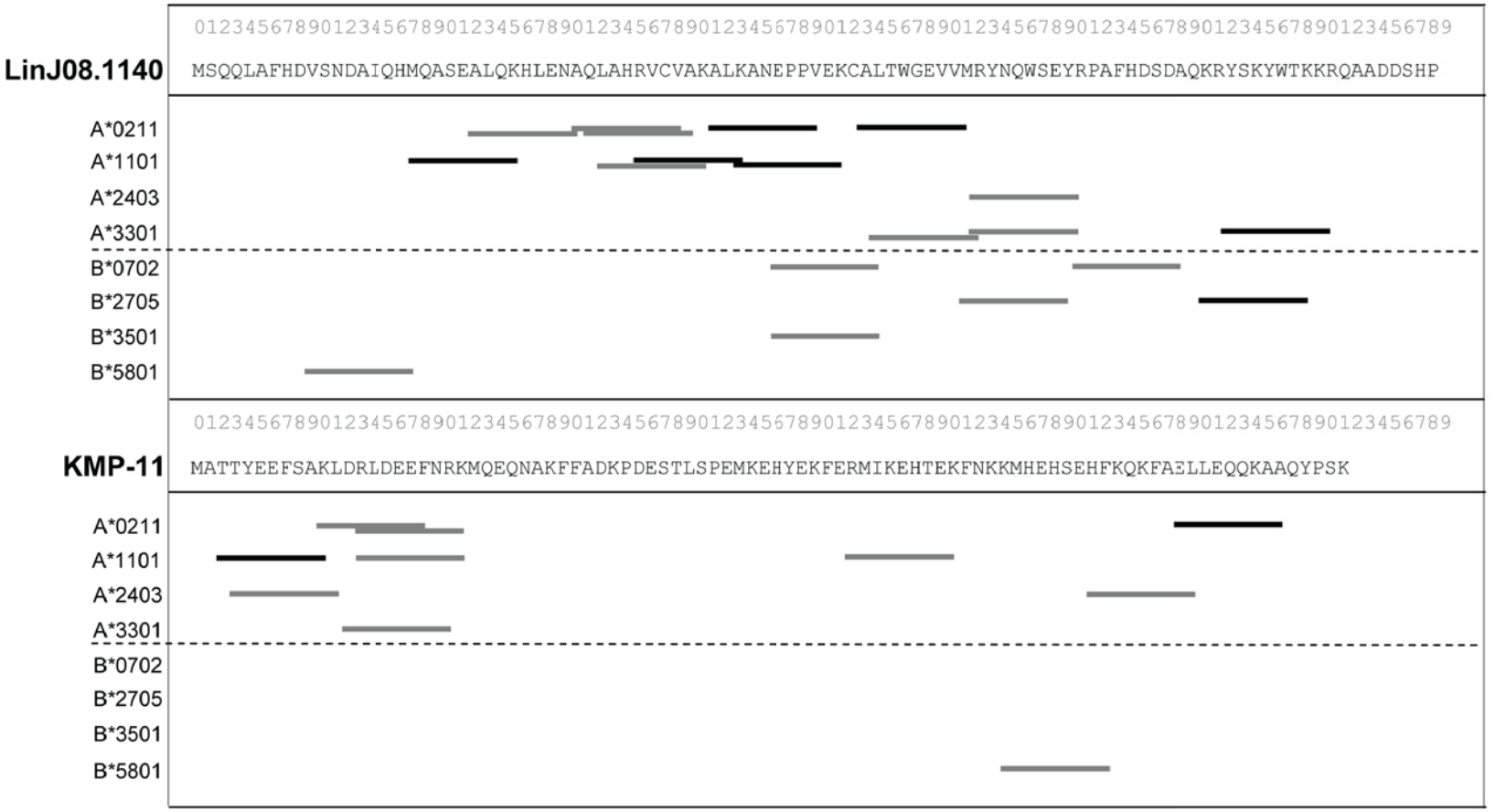

Fig. 3.36: MHC-I epitope prediction for antigens LinJ08.1140 and KMP-11
NetMHC (Url: http://www.cbs.dtu.dk/services/NetMHC/) was used on a selected set of HLA-A and B haplotypes/alleles. Immunogenic peptides are depicted with a bar symbolising a nonamer. Weak binders are shown in grey and strong binders in black.

A*0201 is absent in the Indian population and hence was replaced with the more frequent A*0211.

The result of the epitope prediction is shown in figure 3.36. Analysis of KMP-11 (bottom half) generates a cluster of binding peptides in the N-terminal region of the protein, with one classified as strong binder (black, threshold affinity $\leq$ 50 nM) starting at position 2 and a number of weak binding peptides (grey, threshold affinity $\leq$ 500 nM) in the region 2-21. The C-terminus in comparison is a lot less represented with one strong binder starting at position 78 and a couple of weak binders. This is to a certain degree coherent with experimental data (Basu *et al*., 2007b), which also showed clustered binding regions N-terminal (1-30) and C-terminal (71-90). Interestingly, the majority of peptides found, did match HLA-A haplotypes, while only one weak binder for the chosen HLA-B haplotypes was detected. Due to the coherence with experimental data for KMP-11, it was assumed that a similar analysis with LinJ08.1140 would be valid.

Epitope prediction for LinJ08.1140 using the same haplotypes showed a higher frequency of peptides with strong and weaker binding ability both for HLA-A and HLA-B than KMP-11. A cluster comprising strong binder for HLA-A was found between position 17 and 64, while clusters of weak binders were found for both loci between position 46 and 78. Additionally one strong binder was found for each of the loci starting around amino acid 80. Overall peptide prediction revealed several candidate peptides for $CD8^+$ activation, which in a next step will have to be evaluated in a practical experiment.

4. Discussion

Leishmaniasis is a grossly neglected tropical disease and currently threatens more than 350 million people in 88 countries world wide (Desjeux, 1996). The emergence and spread of HIV, migration due to civil unrest and the phenomenon of global warming contribute to the rise of leishmaniasis (www.who.int/leishmaniasis/en/). Treatment is available, but expensive, toxic or prone to resistance development in parasites, underlining the need for a vaccine. The concept of vaccination is feasible and during the past decades a lot of effort has been invested to understand parameters like immune responses linked to susceptibility or resistance or genetic factors associated with disease. In order to reach the ultimate aim, a vaccine against different forms of leishmaniasis, several strategies such as whole live or dead organism, single antigens in combination with different adjuvant and recombinant delivery systems have been more or less successfully tested (Khamesipour *et al*., 2006; Kedzierski *et al*., 2006; Coler and Reed, 2005; Palatnik-de-Sousa, 2008). Some of these vaccines progressed to clinical studies, but despite all effort no such vaccine has become available yet.

The immunisation with attenuated live pathogens in order to induce a protective immune response in the host has had a long tradition. A major drawback of this approach is that under certain circumstances attenuated strains may gain virulence and become pathogenic again. This is especially in regard to the severity of visceral leishmaniasis not acceptable. A solution to this problem is the use of sub-unit vaccines, instead of the whole organism, with the ambition to induce a protective immune response but not disease. A live vaccine using recombinant, attenuated S. *typhimurium* has been developed and described in this thesis. Furthermore, novel antigen candidates have been identified and evaluated using different *in vivo* models for visceral leishmaniasis. In an attempt to further improve the vaccine effect achieved, outer membrane vesicles have been engineered to inducibly express leishmania antigens and consequently have been tested for their potential to augment antigen specific immune responses.

4.1 *In silico* selection of novel antigen candidates

Several methods to successfully identify putative antigens candidates have been described in the literature. Melby and colleagues, for example, screened a cDNA library which has been generated from *L. donovani* amastigotes. An estimated 30 000 constructs have then been tested in pools and the most protective ones have been further analysed in sub-pools. A major finding was, however, that the sub-pools were less protective. The resulting decrease in protection has been attributed to the loss of synergistic effects of some of the cDNAs. Altogether, the authors describe this method as very laborious and no particular cDNAs have been singled out as vaccine candidates (Melby *et al*., 2000). A further method, involving the fractionation of *L. major* lysate on SDS-Gels and subsequent transfer to PVDF membranes has been described (Zeinali *et al*., 2007). Immunoreactive proteins were identified by incubation of the membrane with serum from patients who recovered from CL and subsequently eluted from the membrane for identification. Serum from healed and immunocompetent patients is also used in immunoproteomics. Lysates of pathogens were separated on two-dimensional electrophoresis and potential antigen candidates are subsequently identified using the anti-sera from patients with healed or active infection and reactive protein spots were then analysed by mass spectrometry. This approach has been used for a number of pathogens such as *Neisseria meningitides*, *Chlamydia*, *H. pylori* and *L. donovani* (Williams *et al*., 2009; Karunakaran *et* a.,l 2008; Haas *et al*., 2002; Forgber *et al*., 2006; Theinert *et al*., 2005). The disadvantage of this method is the requirement of patient serum, which is not always available in excess and more importantly not all protective antigens are immunogenic during disease.

For that reason it was decided to use an *in silico* method to identify and select novel antigen candidates from proteomics data derived by comparative analysis of *L. mexicana* life cycle stages (Paape *et al*., 2008). The majority of these antigens were also detected in another study (table 3.1) using leishmania proteomics but was only published in 2008 (Rosenzweig *et al*., 2008) that is after this thesis project had started. The proteomic approach took advantage of the completion of sequencing projects for *L. major* and *L. infantum*, respectively (Ivens *et al*., 2005; Peacock *et al*., 2007). The sequencing of the *L. major* Friedlin strain led to the prediction of 8272 proteins. Five hundred and nine of these open reading frames have been identified in the

comparative proteome project (Paape *et al*., 2008). This is equivalent to 6.2 percent of the whole proteome and reflects the most abundant proteins present in both life cycle stages.

The *in silico* selection of antigens for vaccine development has been successfully described for the gastric pathogen *H. pylori* (Sabarth *et al*., 2002), which led to the confirmation of HP0231, an antigen which was previously discovered by immunoproteomics (Haas *et al*., 2002) and was later included in a clinical vaccination study (Aebischer *et al*., 2008). According to Sabarth et al. it was presumed that vaccination against abundant protein from pathogens facilitates recognition and thus response by the immune system. For the purpose of the development of an anti-leishmania vaccine, it can be assumed that the 6.2 % of proteins identified in the proteomics study are representing the most abundant proteins.

Genetic diversity clearly exists for *Leishmania* species (Ivens *et al*., 2005; Peacock *et al*., 2007). Therefore it was considered important to favour antigens which are highly conserved among *Leishmania* spp. A comparative sequence analysis using ClustalW has been done for all antigens selected in this thesis (fig 3.2 and supplementary material), which showed a high grade of homology. Furthermore, it is known that vaccination with proteins can induce autoimmune reactions and suboptimal immune responses, due to cross-reactivity with host proteins. Therefore it was of importance that the antigens in question have no homologues in humans or mice. None of the selected antigens showed any homologies. Taken together, five antigens have been selected for this thesis using the *in silico* approach. Two of them have been shown to be partially protective (discussed in the next section), which reflects a high success rate.

4.2 Discovery of two novel protective antigens to vaccinate against *L. major* and *L. donovani*

Five antigens have been selected to be tested in murine models of VL. In addition KMP-11 was chosen to serve as a reference control. Its immunogenic potential as antigen in prophylactic and therapeutic vaccines against VL has been shown in previous studies using KMP-11 expressed by *Toxoplasma gondii* (Ramirez *et al*., 2001), KMP-11 DNA vaccine (Basu *et al*., 2005) and a KMP-11 transfected hybrid-

cell vaccine (Basu *et al*., 2007a). Due to the encouraging results of these studies KMP-11 is currently being developed for clinical studies (T. Aebischer, personal communication).

However, results of vaccination of susceptible BALB/c mice with KMP-11-expressing *S. typhimurium* SL3261 and subsequent challenge with *L. major* were disappointing. Lesion development for the cytosolic expression strain was comparable with the SL3261 carrier control (fig. 3.16A and 3.30A) in several experiments and a reduction in parasitic burden in the spleen was only transient (fig. 3.16B). Interestingly, expression of KMP-11 on the surface of salmonella resulted in an increased parasitic burden compared to control mice (fig. 3.16B). Bhaumik and collegues have shown that vaccination of BALB/c mice with KMP-11-encoding plasmids required the addition of IL-12 to induce a protective immune response, which is in contrast to *L. donovani*, where this kind of vaccination alone was sufficient for protection (Bhaumik *et al*., 2009). This could be an explanation for the failure of KMP-11 formulated as a recombinant salmonella vaccine; hence results from a challenge with *L. donovani* were expected to be more promising. However, data obtained after *L. donovani* challenge reflected and confirmed the previous observations. Mice vaccinated with cytosolic expression strain pcVAC1-KMP showed reduced parasite burdens in spleen and liver at day 28 and 68 after infection (fig. 3.21) compared to sham-immunised mice but no differences were detected in comparison to the carrier control SL3261. Determination of antigen-specific antibody titres in serum showed IgG1 and IgG2a of similar levels in the *L. major* model, which indicated the presence a mixed T_H1/T_H2 response considered to be of advantage for protection against *L. donovani* (Basu *et al*., 2005; Basu *et al*., 2007a; Stager *et al*., 2003a). KMP-11 vaccinated mice from the *L. donovani* study however produced more IgG1 (data not shown), thus were biased towards T_H2 response. Interestingly, mice from both negative control groups (naïve and carrier control SL3261) showed a similar antibody response after infection, with high levels of KMP-11-specific IgG1. Overall these findings suggest that despite promising results for KMP-11 in DNA vaccines and cell-hybrid vaccines, salmonella were presumably not the ideal vaccine carrier for KMP-11, although the novel antigens described in this study were highly protective when delivered by recombinant salmonella. This shows that not only the

nature of the antigen is of importance for vaccine development but also the way of its delivery.

Two out of five antigen candidates initially selected for this study have delivered promising results against both pathogens *L. major* and *L. donovani*. LinJ23.0410 is a small protein of unknown function, which can be expressed in the cytosol as well as the surface of *S. typhimurium*. Mice that had been vaccinated with either variant showed a delayed onset of disease, although surface expression of LinJ23.0410 did deliver better results. Throughout the studies, animals vaccinated with the cytosolic strain, pcVAC1-23.0410 produced antigen-specific IgG2a, whereas IgG1 was below detection limit. After infection however, the ratio IgG1 versus IgG2a varied between different experiments, but overall titres remained relatively low (~100) in comparison with other antigens (~1000 for KMP-11 and LinJ08.1140). Interestingly, the surface expression strain did not elicit any detectable antibody response against LinJ23.0410. Purification experiments have shown this protein to be very hydrophobic. After extraction from inclusion bodies and on-column refolding the protein was eluted in buffer containing imidazole for stabilising purpose, which had to be removed in a dialysis step. This resulted in rapid precipitation of the protein shortly after. In salmonella transportation of this highly hydrophobic protein to the bacterial surface may have resulted in precipitation and thus blockage of the AIDA-pore. Furthermore, pore blockage might be an explanation for the observation that wild type as well as RBS mutant strains showed similar levels of protein expression and bacterial fitness (fig. 3.6 and 3.9) indicating limited toxicity.

The second and particularly protective antigen was LinJ08.1140. Similar to LinJ23.0410 it is a hypothetical protein of unknown function, selected from the comparative proteome analysis. Surface expression of this antigen resulted in a significant reduction of parasitic burden in the spleen and a significant delay in lesion development after challenge with *L. major*. Serum analysis of *L. major* infected mice showed that the initially T_H1 biased immune response was averted towards T_H2, which is in agreement with the observation that vaccination did not fully prevent disease and with the fact that progressive disease in BALB/c mice has been correlated with a T_H2 type immune response. In the *L. donovani* study however, the majority of LinJ08.1140 vaccinated mice show a T_H1 biased response (data not shown) on day 28

which persisted till later stages of infection at day 68 and reflected the fact that disease did not progress in vaccinated mice.
Elevated organ weights for spleen and liver were observed in LinJ08.1140-vaccinated mice at day 28 after infection. This was very likely a result of vaccine-induced inflammation and recruitment of immune cells in response to parasite infection. A similar observation was described in resistant C57BL/6 mice where the effect was attributed to the presence of IL-12, which promoted rapid development of granulomas (Satoskar *et al*., 2000), and in BALB/c mice when function of CTLA-4, a negative regulator of T cell activation was blocked (Murphy *et al*., 1998). This hypothesis was supported by the low LDU numbers measured in spite of the elevated organ weight.
Overall, the level of protection observed for this particular antigen, LinJ08.1140, is considered to be very good. A similar protection status was achieved after vaccination of mice with recombinant antigen HASPB1 (Stager *et al*., 2000). As a result HASPB1 is included in a clinical study which is currently in preparation (T. Aebischer, personal communication).

Most studies using single vaccine antigen formulations did not report sterile immunity, a fact that has also been observed in this thesis. It therefore can be assumed that vaccination against VL will require more than one antigenic target to achieve sterile immunity if ever possible. LinJ08.1140 and LinJ23.0410 were the most protective antigens studied in this work. Like for many other antigens vaccination with these proteins significantly delayed onset of disease and reduced parasitic burden in visceral organs. In order to test for synergy, the respective salmonella vaccine strains emerging from the first *in vivo* evaluation psVAC5-08.1140, psVAC0-23.0410 and pcVAC1-23.0410 had been combined to one vaccine, called “vaccine allstars”. Both antigens seemed to synergize (see figure 3.18), enhancing the vaccination effect against *L. major* significantly. In the *L. donovani* model, no difference between the vaccine allstars and the single expression strain psVAC5-08.1140 was found. Both formulations showed a remarkable reduction in parasitic burden in spleen and liver. Interestingly, an increase in liver and spleen weights, as seen in LinJ08.1140 vaccinated mice, was not observed, when LinJ08.1140 was combined with LinJ23.0410, thus suggesting a counterbalancing effect of the latter with respect to inflammation. Due to the hypothetical nature of both antigens the mechanism leading to this synergy remains unclear. Synergy can be due to additive effects of

immunogenic antigens or to the immuno-modulatory properties of one or more antigens. This kind of effect has been previously demonstrated for Leish-111f, a recombinant poly-protein, proven to be effective against *L. infantum*. Two of its components, TSA and LmSTI1 have been shown to elicit a protective immune response in BALB/c mice when administered with IL-12. The third component, LeIF was only partly protective (Skeiky *et al*., 1998), but found to be a strong T_H1 inducer stimulating the immune system to produce IL-12, IL-18 and IFNγ, thus making the co-administration of IL-12 redundant (Coler and Reed, 2005; Coler *et al*., 2007). Another vaccine, combining protective antigens KMP-11 and HASPB1, is currently in development (T. Aebischer, personal communication). Thus, the novel candidates discovered here may be incorporated into a future multi-component vaccine.

The remaining three antigen candidates tested during the course of this thesis did not confer any significant protection and were not further analysed. Immunisation with salmonella expressing LinJ25.1680 on the surface (psVAC5-25.1680) led to significant exacerbation and rapid disease progression in mice after challenge with *L. major*. Interestingly a second vaccine strain (psVAC0-25.1680) expressing far more LinJ25.1680 antigen under *in vivo* conditions (fig 3.9) did not show exacerbation but similar results as the SL3261 carrier control. The mechanism leading to that observation remains unclear though potentially interesting.

4.3 Vaccine-independent factors to influence and modulate vaccine induced immune response

Unspecific immuno-stimulatory effects such as additional stress, carrier effects and the nutritional status of the mice can modulate the response to vaccination, as observed in this thesis.

Stress can highly influence the immune system and therefore mice are held under equal conditions to allow comparative analysis of immunological data. However, during one experiment, mice suffered additional stress originating from a broken water bottle in that particular cage. Mice from this cage were previously immunised with cytosolic expression strain pcVAC1-23.0410 and were highly protected from *L. major* challenge. Stress related hormones affect major immune functions and are

known to shift immune responses towards an anti-inflammatory T_H2 response. This would mean a major disadvantage for these mice. However, it has also been shown that stress, especially short term, can enhance skin delayed-type hypersensitivity (DTH) reaction in rodents, due to rapid infiltration of immune cells from the blood to the skin (summarised by Elenkov and Chrousos, 2006). This reaction might be a possible mechanism and explanation for the delayed onset of disease after subcutaneous injection of *L. major* parasites.

Dependent of the source of mice; oral administration of the *aro*A$^-$ salmonella strain SL3261 had a major impact on their health. Despite attenuation of the salmonella strain, mice displayed symptoms typical for typhoid fever in mice, such as scrubby fur, weight loss and apathy, and recovery could take up to four weeks. Lowering the CFU of salmonella to 1x 10^9, a dose which is normally well accepted did not lead to any improvement. These severe symptoms were only observed in mice purchased from Harlan UK and it was speculated that nutrition and an altered intestinal flora played a major role in the apparently enhanced virulence of the attenuated vaccine strain. Similar observations were recently discussed by Round and collegues (Round and Mazmanian, 2009). As a result mice which received SL3261 carrier control showed a faster lesion development and disease progression than their PBS counterparts. This might also be an explanation for the observation that immunisation of C57BL/6 mice (Harlan UK) with the vaccine allstars in general did not yield the same level of protection as observed with BALB/c mice (bred at University facilities or purchased from Charles River UK).

Vaccination with live carrier such as salmonella alone can confer protection against experimental infection to certain extends. This effect has been observed in mice from other sources than Harlan UK, which did not encounter salmonella-induced disease as described above. Carrier effects are quite common and are very likely due to unspecific stimulation or the presence of leishmania parasite cross-reactive antigens expressed by the salmonella carrier.

In addition, contamination of the animal facility with murine pathogens occurred during the course of the present studies. One of these pathogens, pinworm *Syphacia obvelata*, is known to induce a T_H2 response with the production of IL-4, IL-13 and specific IgG1 (Michels *et al*., 2006). This, of course, may have modulated the outcome of vaccination experiments against leishmaniasis using *L. major* parasites where a T_H1 response is required for protection.

4.4 Attenuated salmonella as live carrier vaccine against visceral leishmaniasis

The heterologous expression of antigens in attenuated *S. typhimurium* or *S. typhi* for vaccination of mice and humans, respectively, has been studied against a number of pathogens (reviewed by Galen *et al*., 2009). Immune responses are generally dose-dependent (reviewed by Zinkernagel *et al*., 1997) and it has been shown in *S. typhimurium*, that abundantly produced proteins are more likely to be recognized by the immune system and result in activation of specific $CD4^+$ T cells (Rollenhagen *et al*., 2004).

Heterologous antigen expression in salmonella can result in the loss of bacterial fitness due to toxicity, and therewith impair the delivery of antigen and its presentation to the immune system. To overcome this obstacle the *in vivo* inducible P_{pagC} promoter from salmonella was used for this study. The promoter and subsequently antigen expression is activated by low Mg^{2+} concentrations which typically prevail in the phagosome of APCs, where antigen can be processed and directly loaded onto MHC molecules. Expression of the well studied fusion protein GFP_OVA from this promoter showed no effect on bacterial fitness compared to the carrier control, as demonstrated by *in vivo* colonisation assays (Bumann, 2001). Work by Bumann showed that this promoter has a low *in vitro* activity (< 4000 copies of GFP_OVA per cell) compared to a very high activity *in vivo* (> 90 000 copies of the protein per cell), thus reducing rapid plasmid loss *in vivo*. However, here hypothetical proteins of unknown function from leishmania were to be expressed. No information about potential cytotoxicity or their impact on the metabolic burden of the vaccine carrier was available. Over-expression in *E. coli* showed that four proteins accumulated in inclusion bodies, while one; LinJ35.0240 was not expressed at all, thus indicating a negative impact on the host strain. Indeed, the majority of these antigens were not expressible from P_{pagC} in the cytosol of salmonella under *in vivo* conditions, suggesting that expression of these antigens even at reduced levels in RBS mutant strains was not well tolerated.

Bacterial fitness can be increased by fine regulation of protein expression. One possibility to control intracellular expression levels is the selection of promoters of different strengths. The disadvantage of such a system is that promoters may react different in diverse carrier systems thus complicating transfer of results from one

vaccine model to the other (e.g. mouse/*S. typhimurium* to human/*S. typhi*). Therefore, regulation of antigen expression on a translational level seemed to be the better option, as it allowed the use of a single optimised promoter system. This has been done by introduction of point mutations into the ribosomal binding site (RBS) in this study and elsewhere (J. Schroeder, unpublished data). These mutations influence the binding of the ribosome to the mRNA transcript, resulting in altered translation efficiency. A study probing different mutations and their influence on bacterial fitness was previously carried out using *H. pylori* antigen HP0231. Bacterial fitness is determined by counting the CFU in Peyer's patches at day 7, which in general shows the peak colonisation and has been used as a surrogate for total colonisation. The factorial combination of *in vivo* antigen levels and bacterial numbers fit for antigen delivery showed a direct correlation with vaccine-induced immune responses such as specific IgG and T helper cell mediated protection against *H. pylori* challenge (fig. 4.1, Schroeder *et al*., unpublished data).

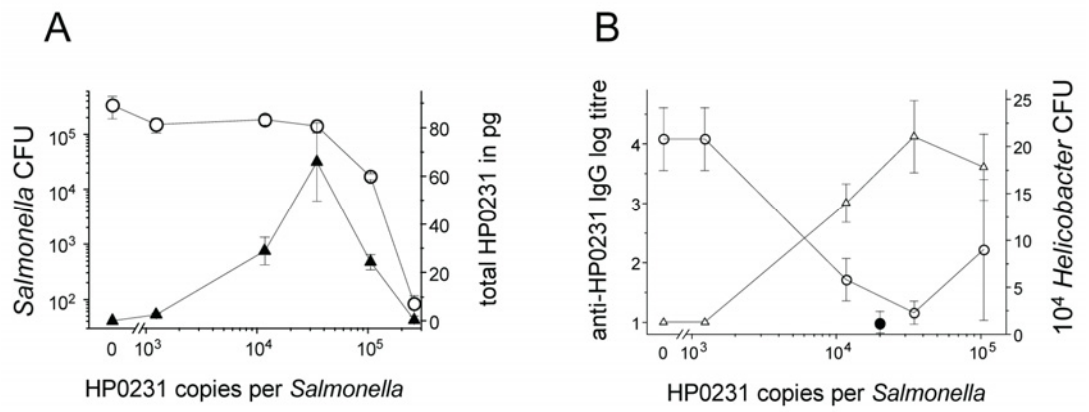

Fig. 4.1: Effect of HP0231 abundance on salmonella antigen delivery, bacterial fitness and immunogenicty

A: Peyer's patches colonization of salmonella expressing various amounts of HP0231 at day 7 post immunization. Based on these colonization data and the estimated *in vivo* HP0231 levels, the total amount of HP0231 was calculated (solid triangles). Means and SEM's for group of 3 to 7 mice are shown. **B**: Serum anti-HP0231 IgG titres (open triangles) seven weeks after immunization with salmonella expressing various HP0231 amounts, and *Helicobacter pylori* stomach loads three weeks after challenge infection (and ten weeks after immunization; open circles).

Therefore this strategy was also applied for the expression of leishmania antigens during this project. For example antigen LinJ23.0410 expression reduced bacterial fitness to a minimum, as no *in vivo* colonisation was detectable at day 7 after oral vaccination. The introduction of a single point mutation in the RBS resulted in a

reduced antigen expression but higher bacterial fitness. This strain, pcVAC1-23.0410 was consequently included in protection studies and was able to reduce parasitic burden in mice. However, antigens LinJ08.1140, LinJ09.1180, LinJ25.1680 and LinJ35.0240 could not be expressed by either wild-type or RBS mutated strains. This was most likely due to toxicity, favouring the outgrowth of strains carrying amber mutations in the cDNA coding the antigen.
However, these antigens were successfully expressed on the surface of salmonella and protection studies resulted in the discovery of psVAC5-08.1140 which was particularly protective. Antigen localisation plays an important role for the induction of an immune response (reviewed by Zinkernagel *et al*., 1997) and surface presentation presumably eases access of proteolytic enzymes in the phagosome of macrophages and loading of peptides onto MHC-II molecules (Verma *et al*., 1995; Hess *et al*., 1996). Furthermore it is known that, like all Gram negative bacteria, salmonella undergo constant membrane shedding. The release of outer membrane vesicles intra- and extracellularly may activate the MHC-II machinery, thus allowing loading of antigenic peptides derived from OMVs.

Leishmania parasites are released into the skin by their sand fly vector. Starting from the site of infection, parasites remain local in case of CL or disseminate to internal organs in case of VL. No sterile immunity after challenge with *L. major* or *L. donovani* was achieved in this study or in any other (Yang *et al*., 1990; Xu *et al*., 1995; McSorley *et al*., 1997; Lange *et al*., 2004) using salmonella-based or other vaccines. This poses the question whether oral vaccination with recombinant typhoid salmonella is a good strategy to address early infection at the site of transmission, or what is needed to improve the vaccine. Oral administration of vaccine strain *S. typhi* Ty21a, registered for human vaccination against typhoid fever and proposed as one of the possible carrier strains for heterologous antigens, was shown to induce mainly gut homing lymphocytes. A vast majority (99 %) of responding lymphocytes carried gut homing receptor $\alpha_4\beta_7$ integrin, whereas L-selectin responsible for homing to peripheral lymph nodes and spleen was found on 42 % and cutaneous lymphocyte antigen (CLA) for skin homing was found only on 1 % of specific circulating T and B lymphocytes (Kantele *et al*., 1997; Salerno-Goncalves *et al*., 2005; Kantele *et al*., 2003; Lundin *et al*., 2002). However, intra-muscular injection of formalin-killed salmonella resulted in L-selectin expression on 86 % of cells, whereas 58 % were

positive for $\alpha_4\beta_7$, indicating that the intra-muscular route of immunisation is able to activate the against VL important systemic immune response (Kantele *et al*., 1997). The same research group investigated the effect of administering multiple doses of salmonella via different routes. Oral re-immunisation led to an up-regulation of skin homing receptor CLA by 34-48 % of reactive lymphocytes independent of the priming route. The group proposed a possible connection between gut and skin in association with food allergies, where skin homing lymphocytes are also up-regulated. They further discuss that the skin area of the body comprises less than 2 m^2, whereas the intestinal surface is estimated to be 400 m^2. Consequently, more specific T cells are required to cover the considerably larger intestinal area. This would be an explanation for the different percentages in homing receptors expressed after salmonella uptake as excessive accumulation of T cells in the skin might be harmful (Kantele *et al*., 2003). For vaccination against VL as demonstrated during this project it can be argued that this one percent skin homing and the 42 % spleen homing is sufficient to significantly reduce parasitic burden in the foot pad (CL) and in the spleen (VL), but that by combining intra-muscular priming with oral boost, the vaccination effect might be further improved.

Protection against leishmaniasis is cell mediated and a relevant vaccine is required to activate antigen-specific $CD4^+$ and $CD8^+$ T cells. Oral vaccination of mice in this study resulted in the activation of $CD4^+$ T cells, as measured by flow cytometry and indirectly by ELISA. The T cell response monitored by cytometry was not compelling, despite indication that psVAC5-08.1140 mediates the activation of IFNγ/TNFα double producing $CD4^+$ T cells. This was most likely due to the low sensitivity of the re-stimulation assay, lacking the addition of fresh antigen presenting cells, or the choice of fluorescence-labelled antibody, especially against IFNγ, or the number of cells acquired, factors which all might improve sensitivity of the assay. Activation of specific $CD8^+$ T cells was not detectable at all and is in coherence with other studies (Yang *et al*., 1990; Lange *et al*., 2004). However, antigen-specific activation of $CD8^+$ T cells against intracellular parasites would be of advantage.

Several possibilities to exploit salmonella live vaccine carrier to target $CD8^+$ T cells have been published. Xiang and collegues used *S. typhimurium* as a DNA-delivering vaccine against cancer. The cancer specific antigen CEA was expressed from a CMV promoter and oral vaccination resulted in the activation of antigen specific $CD8^+$ T

cells and their production of IFNγ, IL-12 and GM-CSF, which more importantly was also detected in the spleen (Xiang *et al*., 2001). This would be a good method for non-cytotoxic antigens like KMP-11 or LinJ23.0410. The most protective antigen of this LinJ08.1140, however, cannot be expressed in the cytosol of *S. typhimurium*. Moreover, expression from mammalian DNA vaccine vector pcDNA3.1 did not result in any detectable protein in HeLa and P815 cells, despite the presence of specific mRNA (data not shown). This characteristic might exclude LinJ08.1140 for DNA vaccination, unless smaller potions such as epitope coding regions are used.

Another attractive method to enhance $CD8^+$ T cell response is the employment of the *S. typhimurium* type III secretions system (T3SS). Genes encoded by salmonella pathogenicity island 2 (SPI-2) are up-regulated upon entry to the phagosome of macrophages. Fusion of heterogeneous proteins or epitopes from *Listeria monocytogenes* to substrates of the T3SS such as SifA and sspH2 resulted in their appearance in the cytosol of host cells 6 to 24 h after oral administration. This led to the activation of $CD8^+$ T cells and also the induction of effector memory T cells and central memory T cells in spleen and blood (reviewed by Panthel *et al*., 2008; Galen *et al*., 2009). An essential requirement for $CD8^+$ activation is the presence of MHC-I epitopes on the antigen. An *in silico* analysis for the protective LinJ08.1140 has therefore been conducted in this thesis. Prediction programs for MHC analysis have a limited reliability. However, a comparative analysis revealed NetMHC to be the most reliable server available online (Lin *et al*., 2008). Simultaneously to LinJ08.1140, KMP-11 was analysed and the results were compared with experimental data for KMP-11 (Basu *et al*., 2007b). Some of the experimentally confirmed epitopes for KMP-11 were also predicted by the server. However, the online prediction seemed to be less sensitive, as not all of the experimental epitopes were recovered. The study by Basu was conducted in Germany and the blood donors used were very likely of Caucasian origin. The MHC I allele A*0201 is highly prevalent in European populations but is completely absent in the Indian population (Mehra, 2000). Presumably, A*0201 specific epitopes were among the experimental data, thus were not included in the *in silico* prediction which used A*0211 instead. Nevertheless, more MHC-I epitopes and especially more strong binding peptides have been predicted for LinJ08.1140 than KMP-11 which is consistent with experimental observations in mouse protection studies. Interestingly no MHC-I epitopes have been

predicted for BALB/c (H-2^d) or C57BL/6 (H-2^b) mice, which may explain the lack of detectable of $CD8^+$ T cell activation.

4.5 Recombinant outer membrane vesicles to augment vaccine-induced immune responses

Outer membrane vesicles are a common feature of Gram-negative bacteria and have been exploited for vaccine development against a number of pathogens such as *N. meningitides*, *Vibrio cholerae* and *S. typhimurium* (Holst *et al*., 2005; Schild *et al*., 2009; Schild *et al*., 2008; Alaniz *et al*., 2007). It has been demonstrated that intra-peritoneal injection of purified salmonella OMVs drastically reduced bacterial burden in mice that have subsequently been intra-veneously challenged with *S. typhimurium* (Alaniz *et al*., 2007). It was reasoned that OMVs carrying respective leishmania antigens could reduce parasitic burden and increase protection against VL. To date only homologous OMVs were produced for vaccination. There is no reason, however, not to produce recombinant OMVs carrying heterologous antigens.

It was assumed that antigens targeted to the surface will automatically appear on OMVs. Therefore the AIDA autotransporter system, already described for salmonella surface expression was used. The *in vivo* inducible P_{pagC} promoter showed very low activity *in vitro* (Bumann, 2001) and was hence replaced by a recently developed plasmid based propionate inducible system using the P_{prpB} promoter (Lee and Keasling, 2005; Lee and Keasling, 2006). Sodium propionate is an inexpensive chemical used as food preservative (Glass *et al*., 2007) and as such is non-toxic in contrast to the for protein induction more commonly used IPTG (Figge *et al*., 1988). Vaccination trials against meningococcal infections in humans have shown that a single dose of OMV corresponding to 25 µg total protein is immunogenic (Gorringe *et al*., 2009). This is close to the 5 to 10 µg doses used in respective mouse experiments (Moe *et al*., 2002; O'dwyer *et al*., 2004). OMVs prepared from one litre of culture routinely yielded about 10 mg of protein, and in that context provide more than 100 immunogenic doses. Therefore, recombinant OMV offer a strategy for the development of affordable vaccine combinations since bacterial culture at industrial scale in large fermenters is economically viable even if products are sold at very low

prices. This is in particular relevant in the context of neglected tropical diseases such as leishmaniasis.

Leishmania parasites are released into the skin after exposure to infected sand flies. Therefore it was decided to administer OMV preparations as a booster injection sub-cutaneously in an attempt to attract $CD4^+$ and $CD8^+$ T cells to the primary site of infections. In this thesis a salmonella-based vaccine against VL has been developed and tested. However, salmonella vaccination predominantly activates gut-homing T cells as discussed before. Antibody responses were determined to characterise the salmonella-prime/OMV-boost concept, although protection against leishmania infection is dependent on the activation of $CD4^+$ and $CD8^+$ T cells. IgG1 and IgG2a responses are dependent of $CD4^+$ T cell help and indicative of IL-4 producing T_H2 or INFγ producing T_H1 responses, respectively, the latter being crucial for anti-parasite immunity (Murray *et al*., 2005). Based on the humoral response, KMP-11 expressing live vaccines induced both T_H1 and T_H2 T cell help. Vaccines expressing LinJ08.1140 and to a far lesser extend LinJ23.0410, however, showed an elevated IgG2a isotype titre indicative for a T_H1 biased response. As mentioned and discussed before, surface expression strain psVAC0-23.0410 did not induce any detectable antigen-specific antibodies. Consistent with a recent KMP-11 vaccine study (Bhaumik *et al*., 2009) and results from this work, immunisation of BALB/c mice with the live vaccine alone did not confer a significant protective local (foot pad) or systemic (spleen) effect against *L. major* challenge. Only animals boosted with the respective OMVs showed a tendency to reduce systemic parasitic burdens, while no improvement was found in psVAC5-08.1140 and pcVAC1-23.0410 vaccinated mice. Interestingly, the only strain where boosting with the respective OMV led to a significant decrease in systemic parasitic burden was the strain expressing LinJ23.0410 on the surface but completely lacking the induction of antigen-specific antibodies. Antibodies have been implicated in exacerbating leishmania infection (Miles *et al*., 2005; Anderson *et al*., 2002; Buxbaum, 2008). The sub-cutaneous route of application has been chosen deliberately not only to attract T cells to the skin but also because the other options intra-venous and intra-peritoneal application would not be viable in human vaccination. It can be speculated that i.p. application in mice, as done by Alaniz and colleagues might have resulted in better protection, since this route targets systemic immunity which is required against VL.

Nevertheless, recombinant OMVs are a versatile platform to express heterologous proteins or antigens for a wide range of applications. For vaccine development different requirements such as safety have to be met. OMV-based vaccines against meningitis have shown clear, but mostly moderate side effects (Gorringe *et al*., 2009; Lennon, 2007; Nokleby *et al*., 2007). Immunisation of individuals in a clinical study resulted in adverse reactions such as pain and erythema. Systemic reactions were in general mild with headache and malaise reported by up to half of the vaccinated individuals. Acceptability of these adverse reactions may depend on the disease targeted but may be less problematic for veterinary vaccines e.g. dogs in the case of VL.

Purified antigens on their own are most often not immunogenic and require the addition of adjuvant to induce an immune response. However, OMVs harbour immuno-stimulatory molecules such as lipoprotein, lipopolysaccharide and peptidoglycan, which are recognised by TLRs 2 and 4 on APCs. Activation of these receptors results in the induction of an inflammatory response and can induce specific T and B cells, thus making the addition of adjuvant redundant. This was shown for OMVs derived from salmonella (Alaniz *et al*., 2007) and *V. cholerae* where, in addition, vesicles were shown to be immunogenic when applied orally (Schild *et al*., 2009; Schild *et al*., 2008). In this thesis it was shown that a single injection with recombinant OMVs, containing approximately 0.5 µg of the vaccination antigens (data not shown), and increased specific antibody titres in mice primed with salmonella vaccine strains 6-40 fold. In a similar study, salmonella primed mice were boosted with 10 µg of antigen absorbed to alum, which resulted in approximately 50 fold higher titres (Londono-Arcila *et al*., 2002). This comparison indicates that recombinant OMVs very potently boosted the antigen specific response.

During the ongoing development of recombinant OMV for this thesis other attempts to derive recombinant OMVs have been reported. For example, fusion constructs of *E. coli* cytotoxin ClyA were engineered to study the process of OMV generation (Kim *et al*., 2008) and outer membrane proteins of pathogenic *N. meningitides* strains were expressed in non-pathogenic neisseria to derive recombinant OMV for vaccination (O'dwyer *et al*., 2004). In addition, OMVs of recombinant *V. cholerae* expressing periplasmic PhoA from *E. coli* were shown to induce PhoA-specific antibodies (Schild *et al*., 2009). This together with the results from this thesis strongly supports the use of recombinant OMVs for vaccination purposes. Compared to these

approaches, however, AIDA provides a more versatile platform, which allows the display of heterologous proteins from different species including Gram-positive bacteria and parasites, and a useful tool for the development of prime-boost reagents.

4.6 Outlook

In this study, two novel antigens have been discovered and prototypic live vaccines expressing these antigens have been developed. These vaccine strains conferred significant protection in susceptible BALB/c mice against challenge with *L. major* and more importantly *L. donovani.* It is clear that further improvements are needed but the results show that live attenuated salmonella are a valid carrier concept to target visceral leishmaniasis.

Dogs are one of the main reservoirs for *L. infantum*, and vaccination would certainly decrease the risk of humans living in close proximity to become accidental hosts. Immunisation of dogs with a DNA vaccine encoding leishmania antigens KMP-11, LACK, gp63 and TRYP failed to induce protection against *L. infantum* (Rodriguez-Cortes *et al*., 2007). Attenuated *S. typhimurium* has been used to vaccinate dogs against salmonellosis (McVey *et al*., 2002) and also in recombinant form against *Echinococcus granulosis* (Chabalgoity *et al*., 2000; Petavy *et al*., 2008). Salmonella expressing antigen LinJ08.1140 showed the highest protection throughout this project and as a first measure the prototypic vaccine developed in this thesis can be used to vaccinate dogs. Studies by Petavy showed that an attenuated *S. typhimurium* isolate from dogs is more immunogenic than mouse adapted strain SL3261. Due to the plasmid based concept applied here, a transfer of the plasmid into a dog adapted *S. typhimurium* strain should be unproblematic.

During this thesis LinJ08.1140 emerged as a particularly protective antigen. It is still classified as hypothetical protein and therefore has not been fully characterised yet. However, for its safe applications in humans and in order to explore its full potential, more research regarding structural and functional properties is necessary. MHC-I epitopes have been predicted in this study but experimental confirmation is required, so that peptides can be used in other epitope-based vaccine formulations.

Leishmania promastigotes have been stained with a polyclonal antibody raised against LinJ08.1140. Fluorescence microscopy revealed a strong signal in dividing parasites, thus suggesting a role of LinJ08.1140 in cell division. This still needs confirmation in amastigotes and further experiments, e.g. Western blots of stage-specific parasite lysates need to be conducted. In addition, a genetic knock-out in leishmania would certainly contribute towards investigating its relevance for the parasite.

The approach to produce recombinant outer membrane vesicles developed here (Schroeder and Aebischer, 2009) may provide an attractive vaccination system in cases where antibodies are of particular importance.

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Supplementary material I

S-1 ClustalW analysis for selected antigens, supplementary to figure 3.2

KMP-11

```
LmjF35.2210__KMP11_          MATTYEEFSAKLDRLDEEFNRKMQEQNAKFFADKPDESTLSPEMKEHYEK 50
L.donovani_KMP11             MATTYEEFSAKLDRLGEEFNRKMQEQNAKFFADKPDESTLSPEMKEHYEK 50
LinJ35_V3.2260__KMP11_       MATTYEEFSAKLDRLDEEFNRKMQEQNAKFFADKPDESTLSPEMKEHYEK 50
LbrM34_V2.2140               MATTYEEFAAKLDRLDEEFNKKMQEQNAKFFADKPDESTLSPEMKEHYEK 50
                             ********:******.****:****************************

LmjF35.2210__KMP11_          FERMIKEHTEKFNKKMHEHSEHFKQKFAELLEQQKAAQYPSK 92
L.donovani_KMP11             FERMIKEHTEKFNKKMHEHSEHFKQKFAELLEQQKAAQYPSK 92
LinJ35_V3.2260__KMP11_       FERMIKEHTEKFNKKMHEHSEHFKQKFAELLEQQKAAQYPSK 92
LbrM34_V2.2140               FERMIKEHTEKFNKKMHEHSEHFKHKFAELLEQQKAAQYPGK 92
                             ************************:***************.*
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LinJ09.1180

```
LinJ09_V3.1180       MLHRVPTLWALRQRAGVTAMAVFGGLSANTGQFACQASPLQQARWASSDAAAPTSNNAQR 60
L.donovani           MLHRVPTLWALRQRAGVTAMAVFGGLSANTGQFACQASPLQQARWASSDAAAPTSNNAQR 60
LmjF09.1120          MLRRVPTLCALRQRAGVTAMAVVGGLSASTGSFACQASPLQQVRWASSDAVAPTSNNAQR 60
LbrM09_V2.1170       MLRRLPTGWALRNHAGVTTMAVAGGLSANPSAFACKAPLLQPARWTSSDAAAPTPVIPQR 60
                     **:*:**  ***::****:*** *****... ***:*. ** .**:****.***.  .**

LinJ09_V3.1180       NNGRNSNVQSQRGRRDAMPPAFDIVHWNDEDITAGHLLRVVHRDGFIVLDYHRQRCVTAE 120
L.donovani           NNGRNSNVQSQRGRRDAMPPAFDIVHWNDEDITAGHLLRVVHRDGFIVLDYHRQRCVTAE 120
LmjF09.1120          NNSRNSSMQSQRGRRDAMPPAFDIVHWNDEDIAAGHLLRVVHRDGFIVLDYHRQRCATAE 120
LbrM09_V2.1170       HNSRSSSVQGQRGRRDDLPPAFDVVHWDDANITAGHLLRVAYRDGFIVLDYHRQRSATSA 120
                     :*.*.*.:*.****** :*****:***:* :*:*******.:*************..*:

LinJ09_V3.1180       LREDGSRAPNPNRAERVVTVTLPPVYVARFLGVLEGRMSKLEVQSRFTNASFSPNAAKGK 180
L.donovani           LREDGSRAPNPNRAERVVTVTLPPVYVARFLGVLEGRMSKLEVQSRFTNASFSPNAAKGK 180
LmjF09.1120          LREDGSRAANPNRAERVVTVTLPPVYVARFLGVLEGRMSKLEVQSRFTNASFSPNAAKGK 180
LbrM09_V2.1170       LREDGSRAVNPNRAERVVTVTLPPVYAARFLGVLEERMSKLEVQSRFTKASFLPNTAKGK 180
                     ******** ****************.******** ************:*** **:****

LinJ09_V3.1180       HHYTLRCTSMKPTTGQIQTVDGADVHEETVEWTVEFDAAESLMLHRFLTQALHYNTGFSR 240
L.donovani           HHYTLRCTSMKPTTGQIQTVDGADVHEETVEWTVEFDAAESLMLHRFLTQALHYNTGFSR 240
LmjF09.1120          HHYTLRCTSMKPTTGQIQTVDGADVHEETVEWTVEFDAAESLMLHRFLTQALHYNTGFSR 240
LbrM09_V2.1170       HHYTLHCTSMKPTTGQIQTADGADVHEETVEWTVEFNAAESLMLHRFLTQALHYNTGFSR 240
                     *****:*************.****************:**********************

LinJ09_V3.1180       KV 242
L.donovani           KV 242
LmjF09.1120          KV 242
LbrM09_V2.1170       KV 242
                     **
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LinJ23.0410

```
LinJ23_V3.0420       -MRRFTSRIAAFAAVPAAEQTRQLHFPISPPPIEIDYLDSDPLEFAVRTEARRWGFDDLQ 59
L.donovani           -MRRFTSRIAAFAAVPAAEQTRQLHFPISPPPIEIDYLDSDPLEFAVRTEARRWGFDDLQ 59
LmjF23.0370          -MRRFTSRIAAFAAVPAAEQTRQLHFPISPPPIEIDYLDSDPLEFAVRTEARRWGFDDLQ 59
LbrM23_V2.0410       MMRRFTSRISAFTAMPAVEQIRQFHFPLTSPPIDIEYLDSDPLEFAVRTEARNWGFDDLQ 60
                      ********:**:*:**.** **:***::.***:*:****************.*******

LinJ23_V3.0420       YMRELAFVRIKNNPSIGDFRNMTPEERRDLFWGSDRQDFFRYITLKLTGHPEHLYHRGW 118
L.donovani           YMRELAFVRIKNNPSIGDFRNMTPEERRDLFWGSDRQDFFRYITLKLTGHPEHLYHRGW 118
LmjF23.0370          YMRELAFVRIKNNPSIGDFRQMTPEERRDLFWGSDRQDFFRYITLKLTGQPEHLYHRGW 118
LbrM23_V2.0410       YMRELAFVRINNNPSIGDFRTMTPEERRDLFWGSDRQDFFRHITYKLTGNPEHLYHRGW 119
                     **********:********* ********************:** ****:*********
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LinJ25.1680

```
LinJ25_V3.1670      MSSEVAIQLTPYPAAFIVEHAKRKKYAAGYILGTVRAEEIVITDFIPHTHKDTEVPNTKA 60
L.donovani          MSSEVAIQLTPYPAAFIVEHAKRKKYAAGYILGTVRAEEIVITDFIPHTHKDTEVPNTKA 60
LmjF25.1610         MSSEVAIQLTPYPAAFIIEHAKRKKYAAGYILGTVRAEEIVITDFIPHTHKDTEVPNTKA 60
LbrM25_V2.2400      ------------------------------------------------------------

LinJ25_V3.1670      HRAELTRRRAAKRRYTTQDLIGWYSAGQPGADLTEEDYQLWCNAPSVIFQGRHCLHLHCE 120
L.donovani          HRAELTRRRAAKRRYTTQDLIGWYSAGQPGADLTEEDYQLWCNAPSVIFQGRHCLHLHCE 120
LmjF25.1610         HRAELTRRRAAKRRYTTQDLIGWYSAGQPGADLTEEDYQLWCNAPSVIFQGRHCLHLHCE 120
LbrM25_V2.2400      ------------------------------------------------------------

LinJ25_V3.1670      MPHEDGTTPKVAWTASVVFEDPADRTLKHIDHRVTLAPMNNLASDVMLSHITSLVLYNGG 180
L.donovani          MPHEDGTTPKVAWTASVVFEDPADRTLKHIDHRVTLAPMNNLASDVMLSHITSLVLYNGG 180
LmjF25.1610         MPHEDGTTPKVTWTASVVFEDPADRTLKHIDHRVTLAPMNNLASDVMLSHITSLVLYNGG 180
LbrM25_V2.2400      MPHEDAATPKVTWTASVVFEDPADRTLKHINHKVTLAPMNNLASDVMLSHITSLSLYNGG 60
                    *****.:****:******************:*:********************* *****

LinJ25_V3.1670      RPFPRSKLQNLDEVAYVASIDHKSSTDAVDEEQRRLERAVAAAQEIVAGGSGGGKEEHEM 240
L.donovani          RPFPRSKLQNLDEVAYVASIDHKSSTDAVDEEQRRLERAVAAAQEIVAGGSGGGKEEHEM 240
LmjF25.1610         RPFPRSKLQNLDEVAYVASIDHKSSTDAVDEEQRRLERAVAAAQEIVTGGSGGGKEEHEM 240
LbrM25_V2.2400      HPFARSKLQNLDEVAYVASIDHKSSTDAVDEVQRRLVRAVEAAQEVVTCGSGGGKEAHEM 120
                    :**.************************** **** *** ****:*: ******* ***

LinJ25_V3.1670      MTAVENFRAIRDEALKRQRDQTGRMDFNSQQFKDALMIKCAATILRREIDQIEHLSTVYG 300
L.donovani          MTAVENFRAIRDEALKRQRDQTGRMDFNSQQFKDALMIKCAATILRREIDQIEHLSTVYG 300
LmjF25.1610         MTAVENFRAIRDEALKRQRDQTGRMDFNSQQFKDALMIKCAATILRREIDQIEHLSTVYG 300
LbrM25_V2.2400      MTAVENFRAIRDEALKRQRDQTGRMDFNSQQFKDALMIKCAATILRREIDQIEHLATVYG 180
                    ************************************************************:****

LinJ25_V3.1670      EDAARRGNATGGENNEKQQQPEKQQQ 326
L.donovani          EDAARRGNATGGENNEKQQQPEKQQQ 326
LmjF25.1610         EDAARRGNATGGENNEKQQQPEKQQQ 326
LbrM25_V2.2400      DDAARRNNATGGENSEKQQ------- 199
                    :*****.*******.****
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LinJ35.0240

```
LinJ35_V3.0140      MLRHSLLRCQMNRAPARPGPTLYGWGRTEQKRRLEYEATESKYHKRDFNKSWDVAGVEQR 60
L.donovani          MLRHSLLRCQMNRAPARPGPTLYGWGRTEQKRRLEYEATESKYHKRDFNKSWDVAGVEQR 60
LmjF35.0140         MLRRSLLRCQVNRAPARPGPTLYGWGRTEQKRRLEYEATESKYHKRDFNKSWDVAGVEQR 60
LbrM34_V2.0180      MLRRSLLRCQVNRAPARPGPTLYGWGRTEQKRRLEYEATESKYHKRDFNKCWDVAGVEQR 60
                    ***:******:****************************************.*********

LinJ35_V3.0140      YSDFMQVRTYFSIGSRWGTWLYNMIQFYVLSMLPIFAFMHFLHKSIEAYDDSIRKAAWW 119
L.donovani          YSDFMQVRTYFSIGSRWGTWLYNMIQFYVLSMLPIFAFMHFLHKSIEAYDDSIRKAAWW 119
LmjF35.0140         YSDFMQVRTYFSIGSRWGTWLYNMIQFYVLSMLPIFAFMHFLHKSIEAYDDSIRKAAWW 119
LbrM34_V2.0180      YSDFMQVRTYFSIGSRWGTWLYNMIQFYVLSMLPIFAFMHFLHKGIEAYDDSIRRAAWW 119
                    ********************************************.*********:****
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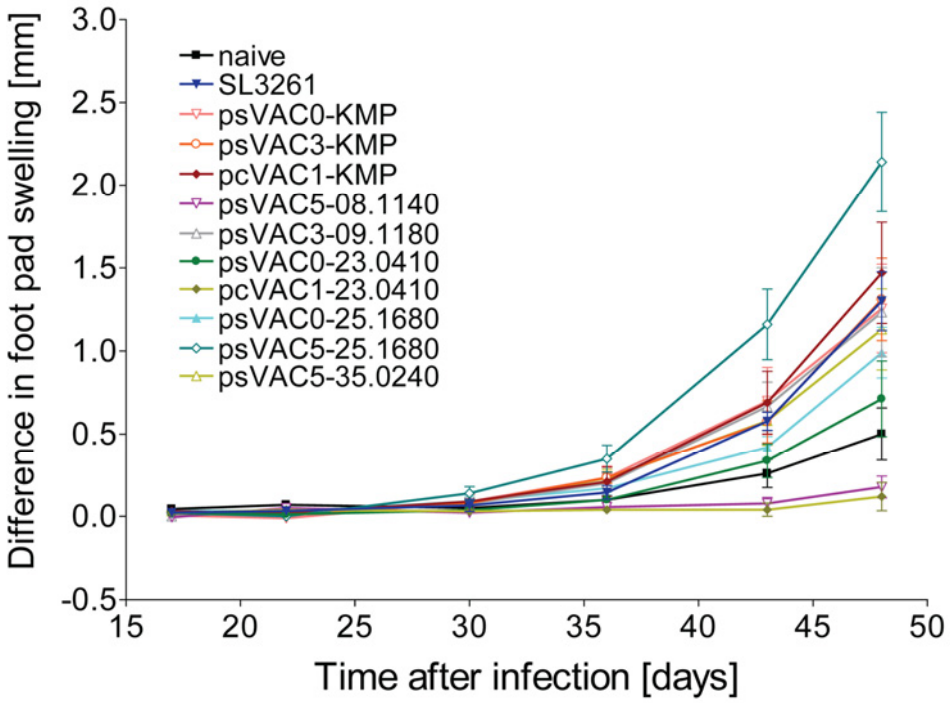
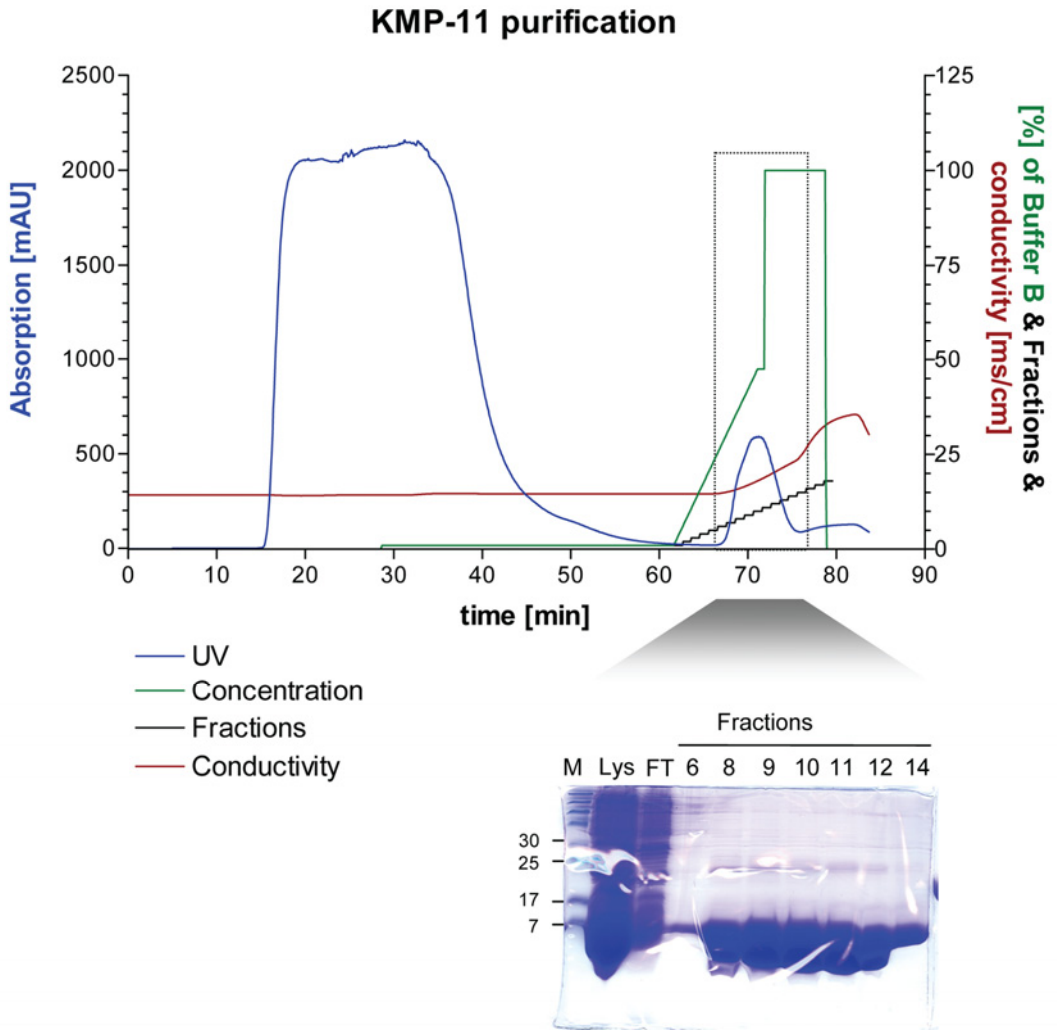
S-2 Evaluation of all vaccine candidates, supplementary to figure 3.16



S-3 Purification of soluble KMP-11, supplementary figure to section 3.24



FT: flow through, Lys: lysate

Publikationen

Schroeder J, Aebischer T. (2009) Recombinant outer membrane vesicles to augment antigen-specific live vaccine responses. *Vaccine*. 2009 Nov 12;27(48):6748-54

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Barrios-Llerena M, Paterou A, Paape D, **Schroeder J**, Aebischer T. The molecular nature of the intracellular habitat of *Leishmania mexicana.* (Manuscript in preparation)

Selbstständigkeitserklärung

Ich erkläre hiermit an Eides statt, dass ich die Dissertation “Selection of novel antigens from Leishmania species and design of live recombinant salmonella vaccines against experimental visceral leishmaniasis“ selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Anna Lehle hat unter meiner Anleitung und Betreuung eine Projektarbeit in unserer Arbeitsgruppe durchgeführt. Ihre Aufgabe war die Bestimmung antigen-spezifischer Antikörper aus Mausserum im Rahmen des Membranvesikelprojekts und einige der daraus resultierenden Rohdaten wurden in meiner Dissertation verwendet.
Die Bestimmung der Vakzineffizienz gegen *Leishmania donovani* wurde in Kollaboration mit Dr. Najmeeyah Brown und Prof. Paul Kaye an der Universität von York durchgeführt. Die mikroskopische Bestimmung der Milz-LDU wurde von Dr. Najmeeyah Brown übernommen.

Juliane Schroeder